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Environmental chemical exposures and human epigenetics

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Every year more than 13 million deaths worldwide are due to environmental pollutants, and approximately 24% of diseases are caused by environmental exposures that might be averted through preventive measures. Rapidly growing evidence has linked environmental pollutants with epigenetic variations, including changes in DNA methylation, histone modifications and microRNAs.

Environmental chemicals and epigenetic changes

All of these mechanisms are likely to play important roles in disease aetiology, and their modifications due to environmental pollutants might provide further understanding of disease aetiology, as well as biomarkers reflecting exposures to environmental pollutants and/or predicting the risk of future disease. We summarize the findings on epigenetic alterations related to environmental chemical exposures, and propose mechanisms of action by means of which the exposures may cause such epigenetic changes. We discuss opportunities, challenges and future directions for future epidemiology research in environmental epigenomics. Future investigations are needed to solve methodological and practical challenges, including uncertainties about stability over time of epigenomic changes induced by the environment, tissue specificity of epigenetic alterations, validation of laboratory methods, and adaptation of bioinformatic and biostatistical methods to high-throughput epigenomics. In addition, there are numerous reports of epigenetic modifications arising following exposure to environmental toxicants, but most have not been directly linked to disease endpoints. To complete our discussion, we also briefly summarize the diseases that have been linked to environmental chemicals-related epigenetic changes.

Keywords Environmental chemicals, epigenetics, disease susceptibility

Background

More than 13 million deaths every year are due to environmental pollutants, and as much as 24% of diseases are estimated to be caused by environmental exposures that can be averted.[1] In a screening promoted by the United States Center for Disease Control and Prevention, 148 different environmental chemicals were found in the blood and urine from the US population, indicating the extent of our exposure to environmental chemicals.[2] Growing evidence suggests that environmental pollutants may cause diseases via epigenetic mechanism-regulated gene

expression changes.[3,4] Dynamic chromatin remodelling is required for the initial steps in gene transcription, which can be achieved by altering the accessibility of gene promoters and regulatory regions.[5] Epigenetic factors, including DNA methylation, histone modifications and microRNAs (miRNAs) (Figure 1), participate in these regulatory processes, thus controlling gene expressions.[6,7] Changes in these epigenetic factors have been shown to be induced by exposure to various environmental pollutants, and some of them were linked with different diseases.[8–10] In this review, we summarize the findings linking environmental chemical exposures with epigenetic alterations, provide some evidence linking such epigenetic changes with diseases (Table 1), and discuss the challenges and opportunities of environmental epigenomics in epidemiologic studies.

Epigenetic factors

DNA methylation

DNA methylation, a naturally occurring modification that involves the addition of a methyl group to the 5′ position of the cytosine ring, is the most commonly studied and best understood epigenetic mechanism.[11] In the human genome, it predominantly occurs at cytosine–guanine dinucleotide (CpG) sites, and serves to regulate gene expression and maintain genome stability.[12]

Environmental studies have shown distinct DNA methylation abnormalities. One commonly reported alteration is an overall genome-wide reduction in DNA methylation content (global hypomethylation) that may lead to reactivation of transposable elements and alter the transcription of otherwise silenced

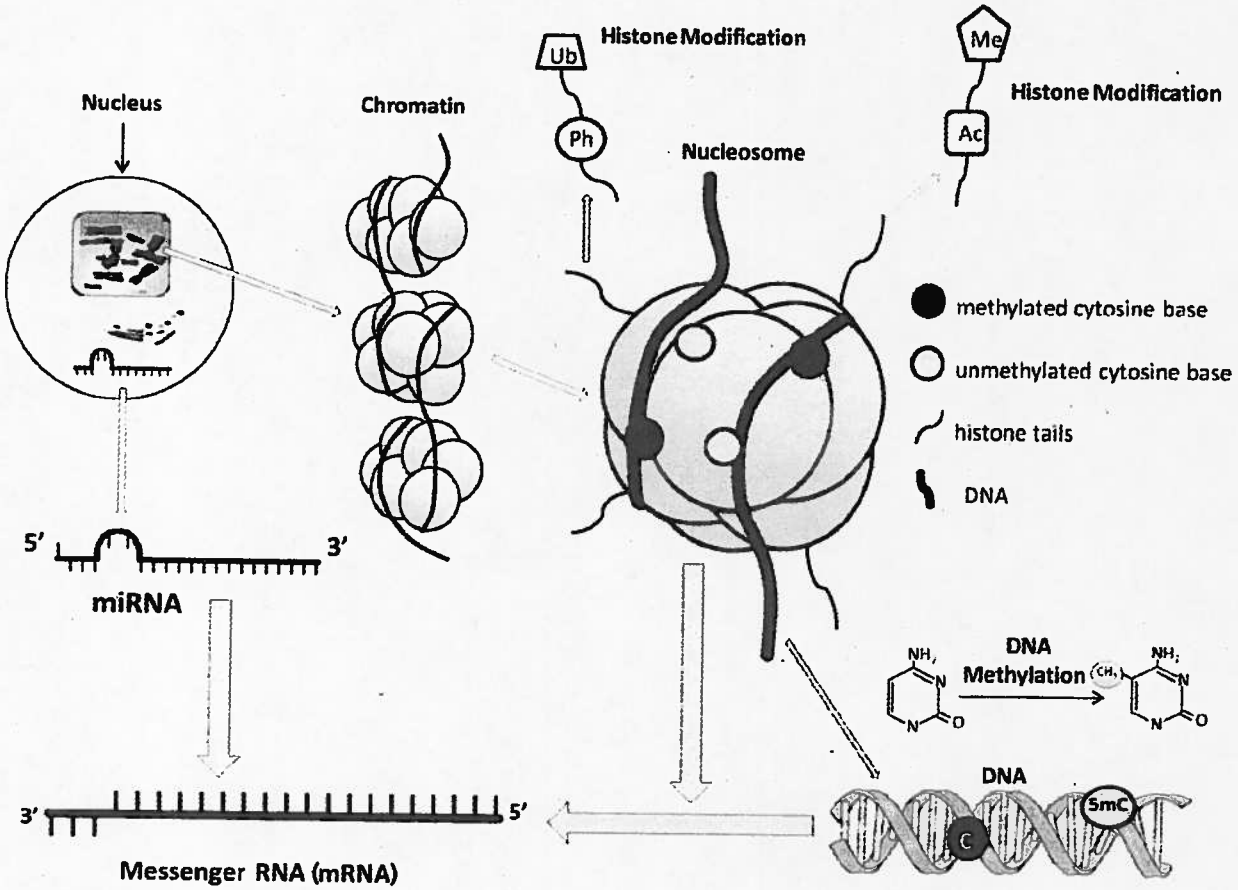

Figure 1 Transcriptional regulation at the epigenetic level. Epigenetic mechanisms, including DNA methylation, histone modifications and miRNAs, regulate chromatin compaction and gene expression. DNA methylation at CpG sites usually suppresses gene expression. Histones are globular proteins that undergo posttranslational modifications, such as Ac, methylation and phosphorylation, thus influencing chromatin structure and gene expression. Active genes are usually characterized by low DNA methylation and highly acetylated chromatin configuration that allow access to transcription factors. miRNAs are a set of small, non-protein-coding RNAs that negatively regulate expression of target genes at the posttranscriptional level by binding to 3′-untranslated regions of target mRNAs

Table 1 Effects of environmental chemicals on epigenetic changes

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
Arsenic	**DNA methylation**			
	Global hypomethylation	*In vitro*	Human HaCaT keratinocytes,[80] human prostate epithelial cell line RWPE-1,[81,82] TRL 1215 rat liver epithelial cell line,[83] V79-Cl3 Chinese hamster cells[226]	Various cancers[227–230] and schizophrenia[231]
	Global hypomethylation	*In vivo*	129/SvJ mice,[84] fisher 344 Rat,[86] homozygous Tg.AC mice,[87] gold-fish,[232] human PBL[233]	Various cancers[227–230] and schizophrenia[231]
	Global hypomethylation and *c-Ha-ras* hypomethylation	*In vivo*	C57BL/6J mice[85]	Various cancers[227–230] and schizophrenia[231]
	Global hypermethylation	*In vivo*	Human PBL[88,89]	Colorectal cancer,[234–236] renal cell carcinoma,[237] acute lymphoblastic leukaemia[238] and bladder urothelial cell carcinoma[239]
	DAPK hypermethylation	*In vitro*	Human uroepithelial SV-HUC-1 cells[90]	Various cancers[240–251]
	P16 hypermethylation	*In vitro*	Human myeloma cell line U266[91]	Various cancers[241,248,250,252–257]
	DBC1, FAM83A, ZSCAN12 and *C1QTNF6* hypermethylation	*In vitro*	Human UROtsa cells[92]	Bladder cancer,[258] breast cancer[259] and malignant lymphoproliferative neoplasms[260]
	P53 hypermethylation	*In vitro*	Human lung adenocarcinoma A549 cells[93]	Breast cancer[261] and hepatoblastoma[262]
	C-myc hypomethylation	*In vitro*	TRL 1215 rat liver epithelial cells[94]	Gastric cancer,[263,264] colon cancer,[263] liver cancer,[207,265,266] kidney cancer[207] and bladder cancer[267]
	C-myc and *c-Ha-ras* hypomethylation	*In vitro*	Syrian hamster embryo cells[95]	Gastric cancer,[263,264] colon cancer,[263] liver cancer,[207,265,266] kidney cancer[207] and bladder cancer[267]
	P16 and *RASSF1* hypermethylation	*In vivo*	A/J mice[96]	Various cancers[241,248,250,252–257,268,269]
	Global hypomethylation and *ER-alpha* hypomethylation	*In vivo*	C3H mice[97]	Various cancers[97,227–230] and schizophrenia[231]
	P53 and *P16* hypermethylation	*In vivo*	Human PBL[98]	Various cancers[241,248,250,252–257,261,262]
	DAPK hypermethylation	*In vivo*	Human bladder, kidney and ureter[99]	Various cancers[240–251]

(continued)

Table 1 Continued

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
	RASSF1A and *PRSS3* hypermethylation	*In vivo*	Human bladder[100]	Lung cancer and prostate cancer[268,269]
	P16 hypermethylation	*In vivo*	Human PBL[270]	Various cancers[241,248,250,252–257]
	P53 hypermethylation	*In vivo*	Human basal cell carcinoma[102]	Breast cancer[261] and hepatoblastoma[262]
	Both hypomethylation and hypermethylation of *VHL*	*In vitro*	Human kidney cells[271]	Renal cell carcinoma[271]
	Histone modification			
	↓H3 acetylation	*In vitro*	UROtsa and URO-ASSC cells[92]	Renal cell carcinomas[272]
	↓H4K16 acetylation	*In vitro*	UROtsa cells[104]	Bladder cancer[273]
	↑H3K14 acetylation	*In vitro*	NB4 cells[105]	Diabetic nephropathy[274]
	↑H3S10 phosphorylation			
	↑H3 phosphorylation	*In vitro*	WI-38 human diploid fibroblast cells[106]	Diabetic nephropathy[274]
	↑H3K9 acetylation	*In vitro*	HepG2 hepatocarcinoma cells[107]	Diabetic nephropathy[274]
	↓H3, H4, H2a, H2b acetylation ↓H3 and H4 methylation	*In vitro*	Drosophila melanogaster tissue culture cell line KC161[103]	Heart disease[275] and traumatic brain injury[276]
	↑H2b methylation			
	↑H3K36 trimethylation	*In vitro*	Human lung carcinoma A549 cells[110]	Diabetic nephropathy,[274] multiple myeloma[277] and prostate cancer[278]
	↓H3K36 dimethylation			
	↑H3K4 dimethylation			
	↑H3K9 dimethylation	*In vitro*	Human lung carcinoma A549 cells[110,279]	Prostate cancer,[278] kidney cancer,[278] lung cancer,[280] HCC[281] and AML[282]
	↓H3K27 trimethylation			
	↑H3K4 trimethylation			
	↑H2AX phosphorylation	*In vitro*	RPMI7951 melanoma cells[112]	Ataxia telangiectasia[283]
	↓H3K18 acetylation	*In vitro*	1470.2 cell line derived from the mouse adenocarcinoma parent line[284]	Prostate cancer[278] and colon cancer[285]
	↓H3R17 methylation			
	miRNAs			
	↑miR-222, ↓miR-210	*In vitro*	TK6 cell line[100]	Various cancers[286–290] and AD[291]
	↓miR-19a	*In vitro*	T24 cell line[115]	Various cancers[292–300]

(continued)

Table 1 Continued

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
Nickel	**DNA methylation**			
	ATF-1, HIF-1, gpt and *Rb* hypermethylation	*In vitro*	G12 cell line[116,117]	Various cancers[301–306]
	P16 hypermethylation	*In vivo*	Mouse histiocytomas[119]	Various cancers[241,248,250,252–257]
	Histone modification			
	↑H3K9 methylation ↓Ac at all four core histones	*In vitro*	Human lung carcinoma A549 cells[123,307]	Heart disease[275] and traumatic brain injury[276]
	↑H3K9 dimethylation ↑H2a, H2b ubiquitylation ↓H3K4 methylation ↓H3K4 acetylation ↓H2a, H2b, H3, H4 acetylation	*In vitro*	Human lung carcinoma A549 cells,[122,124] G12 cells,[116,123,126,128,279] 1HAEo- cell line,[120,121] human (HAE) and rat (NRK) cells,[125] Chinese hamster cell line[127]	Lung cancer,[308] heart disease,[275] chronic glomerular disease[309] and traumatic brain injury[276]
	↓H4K5, H4K8, H4K12, H4K16 acetylation	*In vivo*	Human lung carcinoma A549 cells[130]	Ataxia telangiectasia[310]
	↓H2A, H2B, H3, H4 acetylation (especially in H2BK12 and H2BK20)	*In vitro*	Human airway epithelial 1HAEo- (HAE) cell line[131]	Heart disease[275] and traumatic brain injury[276]
	↑H3 phosphorylation	*In vitro*	Human lung carcinoma A549 cells[132]	Diabetic nephropathy[274]
Cadmium	**DNA methylation**			
	Global DNA hypomethylation	*In vitro*	K562 cell[133]	Colorectal cancer,[234–236] renal cell carcinoma,[237] acute lymphoblastic leukaemia,[238] bladder urothelial cell carcinoma[239]
	Initially induces DNA hypomethylation, prolonged exposure results in DNA hypermethylation	*In vitro*	TRL1215 rat liver cells[134]	Not applicable
	miRNAs			
	↓miR-146a	*In vivo*	Human PBL[137]	Various cancers[311–313]

(continued)

Table 1 Continued

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
Chromium	**DNA methylation**			
	P16 and *hMLH1* hypermethylation	*In vivo*	Human lung[143,144]	Various cancers[241,248,250,252–257,314–316]
	Gpt hypermethylation	*In vitro*	G12 cell line[317]	Not applicable
	Histone modification			
	↓H3S-10 phosphorylation	*In vitro*	Human lung carcinoma A549 cells[279]	Type 2 diabetes,[274] heart disease[275] and traumatic brain injury[276]
	↓H3K4 trimethylation			
	↓H3 and H4 acetylation ↑Dimethylation and trimethylation of H3K9 and H3K4			
	↓H3K27trimethylation and H3R2 dimethylation			
Aluminum	**miRNAs**			
	↑miR-146a	*In vitro*	HN cells[149]	AD,[318,319] cardiac hypertrophy[320] and various cancers[321–328]
	↑miR-9, -128, -125b	*In vitro*	HN cells[329]	AD,[330] neurodegeneration[331] and various cancers[332–335]
Mercury	**DNA methylation**			
	Global hypomethylation	*In vivo*	Brain tissues in polar bear[139]	Neurological disorders[336,337] and various cancer[338]
	Rnd2 hypermethylation	*In vitro*	Mouse embryonic stem cells[140]	neuronal migration defect[339]
Lead	**DNA methylation**			
	Global hypomethylation	*In vivo*	Human PBL,[141] newborn umbilical cord blood samples[142]	Various cancers[227–230] and schizophrenia[231]
Pesticides	**DNA methylation**			
	P53 hypermethylation	*In vitro*	Human lung adenocarcinoma A549 cells[93]	Breast cancer[261] and hepatoblastoma[262]
	Alter DNA methylation in the germ line	*In vivo*	Rat testis[154–156]	Potential effects in the offspring
	Hypomethylation of *c-jun* and *c-myc*	*In vivo*	Mouse liver[158,159]	Gastric cancer,[263,264] colon cancer,[263] liver cancer,[207,265,266] kidney cancer[207] and bladder cancer[267]
	Global hypomethylation (Alu)	*In vivo*	Human PBL[161,162]	Various cancers[227–230] and schizophrenia[231]

(continued)

Table 1 Continued

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
	Both hypomethylation and hypermethylation of *VHL*	*In vitro*	Human kidney cells[271]	Renal cell carcinoma[271]
	Histone modification			
	↑Ac of H3 and H4	*In vitro* and *in vivo*	Immortalized rat mesencephalic/dopaminergic cells (N27 cells)[169]	Parkinson's disease[169]
Air pollution	**DNA methylation**			
	Global hypomethylation	*In vivo*	Human PBL[8]	Various cancers[227–230] and schizophrenia[231]
	iNOS hypomethylation	*In vivo*	Human PBL[173]	Lung cancer[340]
	Global hypermethylation	*In vivo*	C57BL/CBA mice sperm[174]	Colorectal cancer,[234–236] renal cell carcinoma[237], acute lymphoblastic leukaemia[238] and bladder urothelial cell carcinoma[239]
	Hypermethylation of *IFNg* and hypomethylation of *IL4*	*In vivo*	CD4+ T lymphocytes[175]	Asthma[175]
	Histone modification			
	↑H3K4 dimethylation and H3K9 acetylation	*In vivo*	Human PBL[177]	Diabetic nephropathy[274]
	Global hypomethylation (Alu, LINE-1)	*In vivo*	Human buffy coat[317]	Various cancers[227–230] and schizophrenia[231]
	miRNAs			
	↑miR-222	*In vivo*	Human PBL[137]	Various cancers[286–288]
	↑miR-21	*In vivo*	Human PBL[137]	Various cancers[299,341–347]
Benzene	**DNA methylation**			
	Global hypomethylation (Alu, LINE-1)	*In vivo*	Human PBL[8]	Various cancers[227–230] and schizophrenia[231]
	P15 hypermethylation and melanoma antigen-1 (*MAGE-1*) hypomethylation	*In vivo*	Human PBL[165–168,186]	Psoriasis[348] and various cancers[349–360]
	Global DNA hypomethylation	*In vitro*	Human lymphoblastoid cell line TK6[187]	Various cancers[227–230] and schizophrenia[231]
	Hypermethylation of poly (ADP-ribose) polymerases-1 (*PARP-1*)	*In vitro*	Lymphoblastoid cell line F32[188]	Various cancers[188]

(continued)

Table 1 Continued

Environmental chemicals	Epigenetic changes	*In vitro/in vivo*	Tissue/species	Example of diseases potentially associated with the observed changes in epigenetic changes
Bisphenol A	**DNA methylation**			
	Hypomethylation of the Agouti gene and CabpIAP	*In vivo*	Mouse embryo[192]	Mice with hypomethylation of the Agouti gene are obese, diabetic and exhibit increased cancer rates[361,362]
	Hypomethylation of the homeobox gene *Hoxa10*	*In vivo*	CD-1 mice[194]	Not applicable
	Hypermethylation of *LAMP3*	*In vitro*	Breast epithelial cells[195]	Breast cancer[195]
	miRNAs			
	↑miR-146a	*In vitro*	3A placental cells[196]	Cardiac hypertrophy,[320] AD[318,319] and various cancers[321–328]
Dioxin	**DNA methylation**			
	Igf2 hypomethylation	*In vivo*	Rat liver[198]	Russell–Silver syndrome[363–365] and various cancers[366–370]
	Alterations in DNA methylation at multiple genomic regions	*In vivo*	Splenocyte of mice[199]	Not applicable
	miRNAs			
	↑miR-191	*In vivo*	Rat liver[200]	Breast cancer,[342] colorectal cancer[321,371] and gastric cancer[372]
RDX	**miRNAs**			
	↑let-7, miR-15, -16, -26, -181 ↓miR-10b	*In vivo*	Mouse brain and liver[202]	Various cancers[325,373–380]
	↑miR-206, -30, -195	*In vivo*	Mouse brain and liver[202]	Various cancers[342,381–385]
DES	**miRNAs**			
	↓miR-9-3	*In vitro*	Breast epithelial cells[205]	Breast cancer[205]
Drinking water	**DNA methylation**			
	Global hypomethylation *c-myc* hypomethylation	*In vivo*	Mice liver[207,208]	Gastric cancer,[263,264] colon cancer,[263] liver cancer,[207,265,266] kidney cancer[207] and bladder cancer[267]

PBL, peripheral blood leucocytes; HCC, hepatocellular carcinoma; AML, acute myeloid leukaemia; AD, Alzheimer's disease; HN cells, human neural cells; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; DES, diethylstilbestrol.

adjacent genes.[13,14] Global hypomethylation is associated with genomic instability and an increased number of mutational events.[15–18] There are approximately 1.4 million Alu repetitive elements (sequences containing a recognition site for the restriction enzyme *Alu*I)[19] and a half a million long interspersed nucleotide (LINE-1) elements in the human genome that are normally heavily methylated.[20] More than one-third of DNA methylation occurs in repetitive elements.[20] Because of their high representation throughout the genome, LINE-1 and Alu have been used as global surrogate markers for estimating the genomic DNA methylation level in cancer tissues,[20–22] although recent data show lack of correlation with global methylation in normal tissues, such as peripheral blood.[23] Other types of abnormalities that can be induced by environmental pollutants are hyper- or hypo-methylation of specific genes or regions, potentially associated with aberrant gene transcription.[24–27] DNA methylation alterations that directly affect gene expression often occur in the CpG sites located in the promoter regions of the genes. Recent evidence has shown that differentially methylated sites in various cancer tissues are enriched in sequences, termed 'CpG island shores', up to 2 kb distant from the transcription start site.[28] However, to date, gene-specific DNA methylation alterations induced by environmental exposures have been mostly investigated in gene promoter regions. CpG island shores are clearly worthy of further investigation in relation to environmental exposures, but whether they hold such importance in a non-cancer setting remains to be determined.

Histone modifications

In humans, protection and packaging of the genetic material are largely performed by histone proteins, which also offer a mechanism for regulating DNA transcription, replication and repair.[29] Histones are nuclear globular proteins that can be covalently modified by acetylation (Ac), methylation, phosphorylation, glycosylation, sumoylation, ubiquitination and adenosine diphosphate (ADP) ribosylation,[30,31] thus influencing chromatin structure and gene expression.[32,33] The most common histone modifications that have been shown to be modified by environmental chemicals are Ac and methylation of lysine residues in the amino terminal of histone 3 (H3) and H4. Histone Ac, with only a single acetyl group added to each amino acid residue usually, increases gene transcriptional activity;[34–37] whereas histone methylation (Me), found as mono (Me), di-methyl (Me2), and tri-methyl (Me3) group states[38] can inhibit or increase gene expression depending on the amino acid position that is modified.[39–41]

miRNAs

miRNAs are short single-stranded RNAs of approximately 20–24 nucleotides in length that are transcribed from DNA but not translated into proteins. miRNAs negatively regulate expression of target genes at the post-transcriptional level by binding to 3′-untranslated regions of target mRNAs.[42] Each mature miRNA is partially complementary to multiple target mRNAs and directs the RNA-induced silencing complex (RISC) to identify the target mRNAs for inactivation.[43] miRNAs are initially transcribed as longer primary transcripts (pri-miRNAs) and processed first by the RNase enzyme complex, and then by Dicer, leading to incorporation of a single strand into the RISC. miRNAs guide RISC to interact with mRNAs and determine post-transcriptional repression. miRNAs are involved in the regulation of gene expression through the targeting of mRNAs during cell proliferation, apoptosis, control of stem cell self renewal, differentiation, metabolism, development and tumour metastasis.[44,45] Compared with other mechanisms involved in gene expression, miRNAs act directly before protein synthesis and may be more directly involved in fine-tuning of gene expression or quantitative regulation.[46,47] Moreover, miRNAs also play key roles in modifying chromatin structure and participating in the maintenance of genome stability.[48] miRNAs can regulate various physiological and pathological processes, such as cell growth, differentiation, proliferation, apoptosis and metabolism.[42,49] More than 10 000 miRNAs have been reported in animals, plants and viruses by using computational and experimental methods in miRNA-related public databases. The aberrant expression of miRNAs has been linked to various human diseases, including Alzheimer's disease, cardiac hypertrophy, altered heart repolarization, lymphomas, leukaemias, and cancer at several sites.[50–66]

Environmental pollutants and epigenetic alterations

Metals

Heavy metals are widespread environmental contaminants and have been associated with a number of diseases, such as cancer, cardiovascular diseases, neurological disorders and autoimmune diseases.[67,68] In recent years, there has been an increasing appreciation of the roles of molecular factors in the aetiology of heavy metal-associated diseases.[69–71] Several studies showed that metals act as catalysts in the oxidative deterioration of biological macromolecules.[72] Metal ions induce reactive oxygen species (ROS), and thus lead to the generation of free radicals.[72,73] ROS accumulation can affect epigenetic factors.[74–79] Growing data have linked epigenetic alterations with heavy metal exposure.

Arsenic

Evidence has been rapidly increasing that exposure to arsenic (As) alters DNA methylation both globally and in the promoter regions of certain genes.

Upon entering the human body, inorganic As is methylated for detoxification. This detoxification process uses *S*-adenosyl methionine (SAM), which is a universal methyl donor for methyltransferases including DNA methyltransferases (DNMTs) that determine DNA methylation. Thus, it has been shown that As exposure leads to SAM insufficiency and decreases the activity of DNMTs due to the reduction of their substrate. In addition, As has also been shown to decrease *DNMT* gene expression.[80] These As-induced processes may all contribute to global DNA hypomethylation. Arsenic exposure was shown to induce global hypomethylation in a dose-dependent manner in several *in vitro* studies.[80–83] Further, rats and mice exposed to As for several weeks exhibited global hypomethylation in hepatic DNA.[84–87] Nonetheless, evidence in humans is still limited and not completely consistent. In a cross-sectional study of 64 subjects, As level in contaminated water was associated with global DNA hypermethylation in blood mononuclear cells.[88] A global dose-dependent hypermethylation of blood DNA was observed in Bangladeshi adults with chronic As exposure.[89]

Arsenic exposure has also been associated with gene-specific hyper- or hypo-methylation in both experimental settings and human studies.[85,90–101] As exposure has been shown to induce dose-dependent promoter hypermethylation of several tumour suppressor genes, such as *p15*, *p16*, *p53* and *DAPK*, *in vitro* and *in vivo*.[91,93,98,101,102] Furthermore, As exposure-related up-regulation of *ER-alpha*, *c-myc* and *Ha-ras1* gene expression was linked to their promoter hypomethylation in cell lines[94,95] and animal studies.[84,85,97] Evidence in humans is rapidly growing. Toenail As concentration was positively associated with *RASSF1A* and *PRSS3* promoter methylation levels in bladder tumours.[100] Promoter hypermethylation in these two genes was associated with As-induced invasive lung tumours compared with non-invasive tumours.[100] Promoter hypermethylation of *DAPK* was observed in human urocpithelial cells exposed to As,[90] as well as in tumours from 13 of 17 patients living in As-contaminated areas relative to 8 of 21 patients living in As non-contaminated areas.[99] Increased DNA methylation of the *p16* promoter was observed in arseniasis patients when compared with people with no history of As exposure.[101]

Arsenic exposure has also been shown to cause alterations in histone modifications. The earliest evidence on As-induced histone acetylation reductions was in Drosophila.[103] Trivalent As has recently been linked to reduced H3 and H4 lysine 16 (H4K16) acetylation in human bladder epithelial cells.[104] On the other hand, trivalent As exposure has also been shown to increase histone acetylation, which was shown to up-regulate genes related to apoptosis or cell stress response.[105,106] Ramirez *et al.* have reported that As could cause global histone acetylation by inhibiting the activity of histone deacetylases (HDACs).[107] Together, these studies provide evidence that histone acetylation can be dysregulated by As exposure. Early in 1983, As was also shown to induce methylation changes in H3 and H4 in Drosophila.[103] Similar results on H3 were seen in Drosophila Kc 111 cell several years later.[108,109] In recent years, in mammalian cells, arsenite (AsIII) exposure has been associated with increased H3 lysine 9 dimethylation (H3K9me2) and H3 lysine 4 trimethylation (H3K4me3), and decreased H3 lysine 27 trimethylation (H3K27me3).[110,111] As was shown to induce apoptosis by up-regulation of phosphorylated H2AX[112] and cause H3 phosphorylation, which may play important roles in the up-regulation of the oncogenes.[106]

Exposure of human lymphoblast cell line TK-6 to arsenite exhibited global increases in miRNA expression.[113] Arsenic trioxide (As_2O_3) has been used as a pharmacological treatment in acute promyelocytic leukaemia.[114] Cao *et al.*[115] demonstrated that numerous miRNAs were up-regulated or down-regulated in T24 human bladder carcinoma cells exposed to As_2O_3. In particular, miRNA-19a was substantially decreased, resulting in cell growth arrest and apoptosis. The As-related changes in miRNA expression were shown to be reversible when the exposure was removed.[115]

Nickel

Nickel has been proposed to increase chromatin condensation and trigger *de novo* DNA methylation of critical tumour suppressor or senescence genes.[116] In Chinese hamster G12 cells transfected with the *Escherichia coli* guanine phosphoribosyl transferase (*gpt*) gene, nickel was shown to induce hypermethylation and inhibit the expression of the transfected *gpt* gene.[117] An animal study has further shown that nickel induced DNA hypermethylation, altered heterochromatin states and caused gene inactivation, eventually leading to malignant transformation.[118] Govindarajan *et al.*[119] have observed DNA hypermethylation of *p16* in nickel-induced tumours of wild-type C57BL/6 mice, as well as in mice heterozygous for the tumour suppressor *p53* gene injected with nickel compound.

Nickel may cause diseases also via affecting histone modifications. Evidence on nickel-induced histone modifications includes increases of H3K9 dimethylation, loss of histone acetylation in H2A, H2B, H3 and H4, and increases of the ubiquitination in H2A and H2B.[116,120–127] An increase in H3K9 dimethylation and a decrease in H3K4 methylation and histone acetylation was found in the promoter of the *gpt* transgene in G12 cells exposed to nickel.[116,123,128] In mouse PW cells and human cells treated with the HDAC inhibitor trichostatin A, nickel showed a lower capacity to induce malignant transformation.[129]

This finding suggested that gene silencing mediated by histone deacetylation may play a critical role in nickel-induced cell transformation.[129] In addition, nickel has also been shown to induce a loss of histone methylation *in vivo* and decreased activity of histone H3K9 demethylase *in vitro*.[123] Nickel also suppresses histone H4 acetylation *in vitro* in both yeast and mammalian cells.[130,131] Nickel can induce H3 phosphorylation, specifically in serine 10 (H3S10) via activation of the c-jun N-terminal kinase/stress-activated protein kinase pathway.[132]

Cadmium

Cadmium (Cd) has been shown to alter global DNA methylation.[133] Takiguchi *et al.*[134] demonstrated that Cd inhibits DNMTs and initially induces global DNA hypomethylation *in vitro* (TRL1215 rat liver cells). However, prolonged exposure was shown to lead to DNA hypermethylation and enhanced DNMTs activity in the same experiment.[134] Cd can also decrease DNA methylation in proto-oncogenes and promote oncogenes expression that can result in cell proliferation.[133,134]

Transcriptional and post-transcriptional gene regulation is critical in responses to Cd exposure, in which miRNAs may play an important role.[135,136] Bollati *et al.*[137] have recently demonstrated that increased expression of miR-146a in peripheral blood leucocytes from steel workers was related to inhalation of Cd-rich air particles. miRNA-146a expression is regulated by the transcription factor nuclear factor-kappa B, which represents an important causal link between inflammation and carcinogenesis.[138]

Other metals

Mercury (Hg) is widely present in various environmental media and foods at levels that can adversely affect humans and animals. Exposure to Hg has been associated with brain tissue DNA hypomethylation in the polar bear.[139] Arai *et al.*[140] have studied the effects of Hg on DNA methylation status in mouse embryonic stem cells. After 48 or 96 h of exposure to the chemical, they observed hypermethylation of *Rnd2* gene in Hg-treated mouse embryonic stem cells.

Lead is among the most prevalent toxic environmental metals, and has substantial oxidative properties. Long-term exposure to lead was shown to alter epigenetic marks. In the Normative Aging Study, LINE-1 methylation levels were examined in association with patella and tibia lead levels, measured by K-X-Ray fluorescence. Patella lead levels were associated with reduced LINE-1 DNA methylation. The association between lead exposure and LINE-1 DNA methylation may have implications for the mechanisms of action of lead on health outcomes, and also suggests that changes in DNA methylation may represent a biomarker of past lead exposure.[141] In addition, Pilsner *et al.*[142] characterized genomic DNA methylation in the lower brain stem region from 47 polar bears hunted in central East Greenland between 1999 and 2001. They have reported an inverse association between cumulative lead measures and genomic DNA methylation level.

Hexavalent chromium [Cr(VI)] is a mutagen and carcinogen that has been linked to lung cancer and other adverse health effects in occupational studies. Kondo *et al.*[143] found *p16* and *hMLH1* hypermethylation in lung cancer patients with past chromate exposure.[144] *In vitro* experiments on cells exposed to binary mixtures of benzo[a]pyrene (B[a]P) and chromium have shown that B[a]P activates Cyp1A1 transcriptional responses mediated by the aryl hydrocarbon receptor (AhR), whereas chromium represses B[a]P-inducible AhR-mediated gene expression[145,146] by inducing cross-links of histone deacetylase 1–DNA methyltransferase 1 (HDAC1–DNMT1) complexes to the Cyp1A1 promoter chromatin and inhibit histone marks, including phosphorylation of histone H3 Ser-10, trimethylation of H3 Lys-4 and various acetylation marks in histones H3 and H4. HDAC1 and DNMT1 inhibitors or depletion of HDAC1 or DNMT1 with siRNAs blocked the chromium-induced transcriptional repression by decreasing the interaction of these proteins with the Cyp1A1 promoter and allowing histone acetylation to proceed. By inhibiting Cyp1A1 expression, chromium stimulate the formation of B[a]P DNA adducts. These findings may link histone modifications to chromium-associated developmental and carcinogenic outcomes.[147] Chromate exposure of human lung A549 cells has been shown to increase the global levels of di- and tri-methylated histone H3 lysine 9 (H3K9) and lysine 4 (H3K4), but decrease tri-methylated histone H3 lysine 27 (H3K27) and di-methylated histone H3 arginine 2 (H3R2). Most interestingly, H3K9 dimethylation was enriched in the human *MLH1* gene promoter following chromate exposure, and this was correlated with decreased *MLH1* mRNA expression. Chromate exposure increased the protein as well as mRNA levels of G9a, a histone methyltransferase that specifically methylates H3K9. This Cr(VI)-induced increase in G9a may account for the global elevation of H3K9 dimethylation. Furthermore, supplementation with ascorbate, the primary reductant of Cr(VI) and also an essential cofactor for the histone demethylase activity, partially reversed the H3K9 dimethylation induced by chromate. These results suggest that Cr(VI) may target histone methyltransferases and demethylases, which in turn affect both global and gene promoter-specific histone methylation, leading to the silencing of specific tumour suppressor genes.[148]

Recent investigations have demonstrated that aluminum exposure can alter the expression of a number of miRNAs. miR-146a in human neural cells was up-regulated after treatment with aluminium

sulphate. Up-regulation of miR-146a corresponded to the decreased expression of complement factor H, a repressor of inflammation.[149] In addition, a study on aluminium-sulphate-treated human neural cells in primary culture has shown increased expression of a set of miRNAs, including miR-9, miR-125b and miR-128.[150] The same miRNAs were also found to be up-regulated in brain cells of Alzheimer patients, suggesting that aluminum exposure may induce genotoxicity via miRNA-related regulatory elements.[150]

Pesticides

Growing evidence suggests that epigenetic events can be induced by pesticide exposures.[28,151–153] Animal models have shown that exposure to some pesticides, such as vinclozolin and methoxyclor, induces heritable alterations of DNA methylation in male germline associated with testis dysfunction,[154–156] or affects ovarian function via altered methylation patterns.[157] Decreased methylation in the promoter regions of *c-jun* and *c-myc* and increased levels of their mRNAs and proteins were found in livers of mice exposed to dichloro- and trichloroacetic acid.[158,159] Dichlorvos has been demonstrated to induce DNA methylation in multiple tissues in an animal toxicity study.[160] DNA methylation in repetitive elements in blood DNA was inversely associated with increased levels of plasma pesticide residues and other persistent organic pollutants in an Arctic population,[161] a finding later confirmed in a similar study in a Korean population.[162] Whether aberrant DNA methylation represents the link between pesticides and risks of pesticide-related disease, including the excess of cancer risk observed in some epidemiology studies,[163–168] remains to be determined.

Dieldrin, a widely used organochlorine pesticide, has been shown to increase acetylation of core histones H3 and H4 in a time-dependent manner. Histone acetylation was induced within 10 min of dieldrin exposure, suggesting that histone hyperacetylation is an early event in dieldrin-induced diseases. Treatment with anacardic acid, a histone acetyltransferase inhibitor, decreased dieldrin-induced histone acetylation.[169] Dieldrin was further shown to induce histone hyperacetylation in the striatum and substantia nigra in mouse models, suggesting the roles for histone hyperacetylation in dieldrin-induced dopaminergic neuronal degeneration.[170]

Air pollution

Exposure to particulate matter (PM) of ambient air pollution has been associated with increased morbidity and mortality related to cardiovascular and respiratory diseases.[171,172] Black carbon, a component of PM derived from vehicular traffic, has been linked to decreased DNA methylation in LINE-1 repetitive elements in 1097 blood DNA samples of elderly men in the Boston area. Additional evidence for PM effects on DNA methylation stemmed from an investigation of workers in a steel plant with well-characterized exposure to PM with diameters of $<10\,\mu m$ (PM_{10}). Methylation of inducible nitric oxide synthase gene promoter region was decreased in blood samples of individuals exposed to PM_{10} after 3 days of work in the foundry when compared with baseline.[173] In the same study, methylation of Alu and LINE-1 was negatively related to long-term exposure to PM_{10}.[173] In contrast, an animal experiment on mice exposed to air particles collected from a steel plant showed global DNA hypermethylation in sperm genomic DNA, a change that persisted after removal of environmental exposure.[174] Inhaled diesel exhaust particles' exposure and intranasal *Aspergillus fumigatus* induced hypermethylation of several sites of the *interferon gamma* (*IFNγ*) promoter and hypomethylation at a CpG site of the *IL-4* promoter in mice. Altered methylation of promoters of both genes was correlated with changes in IgE levels.[175,176]

We recently also associated PM exposure with histone modifications in the above-mentioned steel workers with high exposure level to PM.[177] In this study, exposure duration (years of work in the foundry) was associated with increased H3K4me2 and H3K4ac in blood leucocytes.[177] In the same study, we showed that exposure to metal-rich PM induced rapid changes in the expression of two inflammation-related miRNAs, i.e. miR-21 and miR-222, measured in peripheral blood leucocytes.[178] Using microarray profiling, Jardim *et al.*[172] have shown extensive alterations of miRNA expression profiles in human bronchial epithelial cells treated with diesel exhaust particles. Out of 313 detected miRNAs, 197 were either up- or down-regulated by at least 1.5-fold.[172]

Benzene

Benzene is an environmental chemical that has been associated with increased risk of haematological malignancies, particularly with acute myeloid leukaemia and acute nonlymphocytic leukaemia.[179–184] Benzene ranks among the top 20 chemicals for production volume in USA.[185] Our results from a study of police officers and gas-station attendants have shown that low-dose exposure to airborne benzene is associated with alterations in DNA methylation in blood DNA of healthy subjects that resemble those found in haematological malignancies,[165–168,186] including hypomethylation of LINE-1 and Alu repetitive elements, hypermethylation of *p15* tumour suppressor gene and hypomethylation of *MAGEA1* (melanoma-associated antigen 1 gene). Consistently, reductions of global DNA methylation has been recently shown in human lymphoblastoid cells treated with benzene metabolites.[187] *In vitro* experiments have also shown that benzene exposure induces hypermethylation of

poly (ADP-ribose) polymerases-1 (*PARP-1*), a gene involved in DNA repair.[188]

Bisphenol A

Bisphenol A (BPA) is an endocrine disruptor with potential reproductive effects, as well as a weak carcinogen associated with increased cancer risk in adult life through fetal exposures.[189,190] BPA is widely used as an industrial plasticizer in epoxy resins for food and beverage containers, baby bottles and dental composites.[191] Dolinoy *et al.*[192] reported that periconceptional exposure to BPA shifted the coat colour distribution of the viable yellow agouti (A^{vy}) mouse offspring toward yellow by decreasing CpG methylation in an intracisternal A particle (IAP) retrotransposon upstream of the Agouti gene.[193] In this animal model, the yellow-coat phenotype is associated with increased cancer rates, as well as with obesity and insulin resistance. In the same set of experiments, maternal dietary supplementation, with either methyl donors like folic acid or the phytoestrogen genistein, blunted the effect of BPA on IAP methylation and prevented the coat colour change caused by BPA exposure.[192] In pregnant CD-1 mice treated with BPA, Bromer *et al.*[194] found decreased methylation and increased expression of the homeobox gene *Hoxa10*, which controls uterine organogenesis. In breast epithelial cells treated with low-dose BPA, gene expression profiling identified 170 genes with expression changes in response to BPA, of which expression of lysosomal-associated membrane protein 3 (*LAMP3*) was shown to be silenced due to DNA hypermethylation in its promoter.[195]

In a recent study by Avissar-Whiting *et al.*,[196] an elevated expression of miR-146a was observed in BPA-treated placental cell lines and miR-146a expression was associated with slower cell proliferation and higher sensitivity to the bleomycin-induced DNA damage.

Dioxin

Dioxin is a compound that has been classified as a human carcinogen by the International Agency for Research on Cancer. As dioxin is only a weak mutagen, extensive research has been conducted to identify potential mechanisms contributing to carcinogenesis. One proposed pathway to carcinogenesis is related to the powerful dioxin-induced activation of microsomal enzymes, such as CYP1B1, that might activate other procarcinogen compounds to active carcinogen. The capability of dioxin to induce *CYP1B1* has been recently shown *in vitro* to depend on the methylation state of the *CYP1B1* promoter.[197] Also, dioxin was shown to reduce the DNA methylation level of *Igf2* in rat liver.[198] Recently, alterations in DNA methylation at multiple genomic regions were identified in splenocytes of mice treated with dioxin, a finding potentially related to dioxin immunotoxicity.[199] In a xenograft mouse model of hepatocellular carcinoma, Elyakim *et al.*[200] have also found that dioxin up-regulated miR-191. In the same study, inhibition of miR-191 inhibited apoptosis and decreased cell proliferation, suggesting that increased miR-191 expression may contribute to determine dioxin-induced carcinogenicity.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX, also known as hexogen or cyclonite)

Hexahydro-1,3,5-trinitro-1,3,5-triazine (commonly known as RDX, the British code name for Royal Demolition Explosive) is an explosive polynitramine and common ammunition constituent used in military and civil activities. Although most of this environmental pollutant is found in soils, RDX and its metabolites are also found in water sources.[201] Exposure to RDX and its metabolites could cause neurotoxicity, immunotoxicity and cancers.[202] Zhang *et al.*[202] have recently evaluated the effects of RDX on miRNA expression in mouse brain and liver. In this study, out of 113 miRNAs, 10 were up-regulated and 3 were down-regulated. Most of the miRNAs that showed altered expression, including let-7, miR-17-92, miR-10b, miR-15, miR-16, miR-26 and miR-181, were found to regulate toxicant-metabolizing enzymes, as well as genes related to carcinogenesis and neurotoxicity.[202]

Diethylstilbestrol

Diethylstilbestrol (DES) is a synthetic oestrogen that was used to prevent miscarriages in pregnant women between the 1940s and the 1960s.[203] A moderate increase in breast cancer risk has been shown both in daughters of women who were treated with DES during pregnancy, as well as in their daughters.[204] Hsu *et al.*[205] have demonstrated that the expression of 82 miRNAs (9.1% of the 898 miRNAs evaluated) were altered in breast epithelial cells when exposed to DES. In particular, the suppression of miR-9-3 expression was accompanied by promoter hypermethylation of the miR-9-3 coding gene in DES-treated epithelial cells.[205]

Chemicals in drinking water

Chlorination by-products are formed as a result of the water chlorination for anti-fouling purposes. Various chlorination by-products in drinking water, such as triethyltin,[206] chloroform[207] and trihalomethanes,[208] have been questioned for potential adverse health effects.[209] These chemicals have been shown to induce certain epigenetic changes. Rats that were chronically intoxicated with triethyltin in drinking water showed development of cerebral oedema as well as an increase of phosphatidylethanolamine-*N*-methyltransferase activities. This increased

methylation might be a compensatory mechanism for counteracting the membrane damages induced by triethyltin.[206] Chloroform, dichloroacetic acid (DCA) and trichloroacetic acid (TCA), three liver and kidney carcinogens, are by-products of chlorine disinfection found in drinking water.[210,211] Mice treated with DCA, TCA and chloroform show global hypomethylation and increased expression of c-*myc*, a proto-oncogene involved in liver and kidney tumours.[207] Trihalomethanes (chloroform, bromodichloromethane, chlorodibromomethane and bromoform) are regulated organic contaminants in chlorinated drinking water. In female B6C3F1 mouse liver, trihalomethanes demonstrated carcinogenic activity. Chloroform and bromodichloromethane decreased the level of 5-methylcytosine in hepatic DNA. Methylation in the promoter region of the *c-myc* gene was reduced by the trihalomethanes, consistent with their carcinogenic activity.[208]

Environmental epigenomics: challenges and opportunities for epidemiologic studies

The studies reviewed in this article have demonstrated the potential effects of environmental pollutants on the epigenome. Several of the epigenomic changes observed in response to environmental exposures might be mechanistically associated with susceptibility to diseases (Table 1). Further studies of epigenetic mechanisms in disease pathogenesis, including the role of epigenetics in the developmental origins of health and disease, their relationships with environmental exposures and the pathways associated with the disease phenotype may help develop preventive and therapeutic strategies.

Epigenetics and developmental origins of health and disease

During embryogenesis, epigenetic patterns change dynamically to adapt embryos to be fit for further differentiation.[7] Two waves of epigenetic reprogramming, which take place at the zygote stage and during primordial germ cells formation, accompany mammalian development.[212]

Experiments on mice carrying the A^{vy} have demonstrated that embryo life is a window of exquisite sensitivity to the environment. In viable yellow (A^{vy}/a) mice, transcription originating in a IAP retrotransposon inserted upstream of the agouti gene (A) causes ectopic expression of agouti protein, resulting in yellow fur, obesity, diabetes and increased susceptibility to tumours.[213] BPA is a high-production-volume chemical used in the manufacture of polycarbonate plastic. In utero or neonatal exposure to BPA is associated with higher body weight, increased breast and prostate cancer and altered reproductive function.

Additional experimental studies have suggested epigenetic mechanisms as potential intermediates for the effects of prenatal exposures to pesticides such as vinclozolin and methoxyclor,[154] as well as of other conditions such as nutritional supplies of methyl donors.[192] Evidence has also been accumulating in humans. Investigations of candidate loci among individuals prenatally exposed to poor nutrition during the Dutch famine in 1944–45 indicate that epigenetic changes induced by prenatal exposures may be common in humans, although they appear to be relatively small and greatly dependent on the timing of the exposure during gestation.[214,215] Based on findings of changes in DNA methylation in subjects exposed to the Dutch famine, Heijmans *et al.*[216] have suggested that the epigenome may represent a molecular archive of the prenatal environment, via which the in-utero environment may produce serious ramifications on health and disease later in life. Terry *et al.*[217] found that prenatal exposure to cigarette smoke was associated with increased overall blood DNA methylation level in adulthood. Other examples include decreased LINE-1 and Sat 2 methylation level in adults and children prenatally exposed to smoking,[218] and global DNA hypomethylation in newborns with utero exposures of maternal smoking.[219] In addition to these DNA methylation changes, Maccani *et al.*[220] have recently observed that miR-16, miR-21 and miR-146a were down-regulated in cigarette smoke-exposed placentas compared to controls.

Additional well-conducted epigenetic studies are now warranted to generate a catalogue of regions that are sensitive to the prenatal environment and may reflect developmental influences on human disease.

Can we develop epigenomic biosensors of past exposures?

An important property of epigenomic signatures is that, because they can be propagated through cell division even in cells with high turnover, they can persist even after the exposure is removed. In addition, as discussed above, an individual's epigenome may also reflect his/her prenatal environmental exposure experience. Thus, epigenomic profiling of individuals exposed to environmental pollutants might provide biosensors or molecular archives of one's past or even prenatal environmental exposures. Using epigenomics, exposure assessment might be brought to research investigations and preventive settings where repeated collections of exposure data might be unfeasible or exceedingly expensive. Further research is needed to establish how rapid are the changes induced by environmental pollutants, as well as whether they accumulate in response to repeated or continuous exposure and how long they persist after the exposure is removed.

What are suitable study designs and approaches for environmental epigenomics?

The field of environmental epigenetics has evolved rapidly in the past several years. As research applications grow, investigators will be facing several difficulties and challenges. Some studies have produced inconsistent results on same pollutants. Several factors may contribute to the inconsistencies. Epigenetic alterations are tissue specific.[221] It is conceivable that the same environmental pollutant may produce different epigenetic changes in different tissues, and even within the same tissue on different cell types. Larger studies with well-defined exposure information that allows examining epigenetic changes across different tissues are needed. Different study design, small sample size and different laboratory methods may also be major causes for the inconsistency. Replicating results and identifying the sources of variability across studies is a major challenge for epigenetic investigations. Because epigenetic markers change over time, disease outcomes are prone to reverse causation, i.e. an association between a disease and an epigenetic marker may be determined by an influence of the disease on the epigenetic patterns, rather than vice versa.[222] Although epigenetic alterations that were found to be induced by or associated with environmental pollutants were also found in various diseases, almost no study has examined the sequence of exposures, epigenetic alterations and diseases.

Longitudinal studies with prospective collection of objective measures of exposure, biospecimens for epigenetic analyses and preclinical and clinical disease outcomes are needed to appropriately establish causality. Existing prospective epidemiology investigations might provide resources for mapping epigenomic changes in response to specific chemicals. However, cohort studies in which biospecimens have been previously collected for genetic or biochemical studies might pose several challenges. Most studies have collected biospecimens, such as blood, urine or buccal cells, which might not necessarily participate in the aetiology of the disease of interest. Methods of collection and processing (e.g. whole blood vs buffy coat) might modify the cell types stored, thus potentially impacting on epigenetic marks. In addition, high-coverage methods providing high-dimensional data on DNA methylation, histone modifications and miRNA expression are increasingly used in human investigations.

Albeit epigenetic mechanisms have properties that make them ideal molecular intermediates of environmental effects, the proportion of the effects of any individual environmental exposure that might be mediated through epigenetic mechanisms is still undetermined. Epidemiology and statistical approaches, including well-designed prospective studies and advanced statistical methods for causal inference are urgently needed. Similarly to genomic studies,[223] epidemiological causal reasoning in epigenomics should include careful consideration of knowledge, data, methods and techniques from multiple disciplines.

The potential interactions between different forms of epigenetic modification

Most studies in environmental epigenetics have separately evaluated only one of the types of the epigenetic marks, i.e. DNA methylation, histone modifications or miRNA expression. However, epigenetic marks are related by an intricate series of interactions that may generate a self-reinforcing cycle of epigenetic events directed to control gene expression.[224] For instance, histone deacetylation and methylation at specific amino acid residues contribute to the establishment of DNA methylation patterns. miRNA expression is controlled by DNA methylation in miRNA encoding genes, and, in turn, miRNAs have been shown to modify DNA methylation.[225] Future studies that include comprehensive investigations of multiple epigenetic mechanisms might help elucidate the timing and participation of DNA methylation, histone modifications and miRNAs to determine environmental effects on disease development.

Can epigenomics be used for prevention?

One major objective of epidemiology investigations is to provide the groundwork for future preventive interventions. Numerous clinical and preclinical studies showed that most of the epigenetic changes are reversible, which offers novel insights to develop new preventive and therapeutic strategies that might take advantage of molecules that modify the activities of epigenetic enzymes, such as DNMTs and HDACs, as well as of the growing field of RNAi therapeutics. Drugs have been designed and developed that produce functional effects, such as histone acetylation and DNA hypomethylation that might be used to restore the normal transcription level of genes. Future epidemiology studies have a unique opportunity to evaluate whether the effects of environmental exposures on the epigenome are mitigated by positive changes in lifestyles, or worsened by the interaction with other risk factors. Future epigenomic research may provide information for developing preventive strategies, including exposure reduction, as well as pharmacological, dietary or lifestyle interventions.

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KEY MESSAGES

- Rapidly growing evidence has linked environmental pollutants with epigenetic variations, including changes in DNA methylation, histone modifications and microRNAs.
- Some of such epigenetic changes have been associated with various diseases.
- Further studies of epigenetic mechanisms in disease pathogenesis, their relationships with environmental exposures and related pathways are needed for the development of preventive and therapeutic strategies.
- Future epidemiology studies on environmental pollutants and epigenome face several challenges.

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As metabolism depletes SAM, AsMT outcompetes DMNT → hypomethylation (liver)

Oxidative stress → GSH depletion → SAM depletion through trans-sulfurase shunt (other tissues)

IAs or metabolites + DMNT → inactivation or inhibition

Induction of DMNT at low doses → hypermethylation

Hypomethylation → chromosome defects, genetic instability

Hypermethylation → reduced expression of tumor suppressor genes

Changes in gene expression (+ other stressors?) → tumor initiation and growth

An Emerging Role for Epigenetic Dysregulation in Arsenic Toxicity and Carcinogenesis

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Background: Exposure to arsenic, an established human carcinogen, through consumption of highly contaminated drinking water is a worldwide public health concern. Several mechanisms by which arsenical compounds induce tumorigenesis have been proposed, including oxidative stress, genotoxic damage, and chromosomal abnormalities. Recent studies have suggested that epigenetic mechanisms may also mediate toxicity and carcinogenicity resulting from arsenic exposure.

Objective: We examined the evidence supporting the roles of the three major epigenetic mechanisms—DNA methylation, histone modification, and microRNA (miRNA) expression—in arsenic toxicity and, in particular, carcinogenicity. We also investigated future research directions necessary to clarify epigenetic and other mechanisms in humans.

Data sources and synthesis: We conducted a PubMed search of arsenic exposure and epigenetic modification through April 2010 and summarized the *in vitro* and *in vivo* research findings, from both our group and others, on arsenic-associated epigenetic alteration and its potential role in toxicity and carcinogenicity.

Conclusions: Arsenic exposure has been shown to alter methylation levels of both global DNA and gene promoters; histone acetylation, methylation, and phosphorylation; and miRNA expression, in studies analyzing mainly a limited number of epigenetic end points. Systematic epigenomic studies in human populations exposed to arsenic or in patients with arsenic-associated cancer have not yet been performed. Such studies would help to elucidate the relationship between arsenic exposure, epigenetic dysregulation, and carcinogenesis and are becoming feasible because of recent technological advancements.

Key words: arsenic carcinogenesis, arsenical compounds, DNA methylation, epigenetics, histone modification, microRNA. *Environ Health Perspect* 119:11–19 (2011). doi:10.1289/ehp.1002114 [Online 2 August 2010]

The International Agency for Research on Cancer (IARC) classified arsenic, a toxic metalloid, as a group 1 carcinogen > 20 years ago (IARC 1987). It is widely accepted that exposure to arsenic is associated with lung, bladder, kidney, liver, and nonmelanoma skin cancers (IARC 2004; Pershagen 1981; Smith et al. 1992; Smith and Steinmaus 2009). High levels of arsenic have also been associated with the development of several other diseases and deleterious health effects in humans, such as skin lesions (dyspigmentation, keratosis), peripheral vascular diseases, reproductive toxicity, and neurological effects (Abernathy et al. 1999).

Exposure to arsenic typically results from either oral arsenic consumption through contaminated drinking water, soil, and food, or arsenic inhalation in an industrial work setting. Arsenic-contaminated drinking water has been associated with increased mortality of bladder and lung cancer in Chile (Marshall et al. 2007) and with increased mortality of both noncancerous causes and cancers in Bangladesh (Sohel et al. 2009). In the human arsenic metabolic pathway, inorganic pentavalent arsenic (As^{V}) is converted to trivalent arsenic (As^{III}), with subsequent methylation to monomethylated and dimethylated arsenicals (MMA, DMA, respectively) (Drobna et al. 2009). The general scheme is as follows:

$$As^{V}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + Me^{+} \rightarrow MMA^{V}O_3^{2-} + 2e \rightarrow MMA^{III}O_2^{2-} + Me^{+} \rightarrow DMA^{V}O_2^{-} + 2e \rightarrow DMA^{III}O^{-}.$$

Methylated arsenicals, especially MMA^{III}, are considered more toxic than inorganic As^{III} both *in vivo* (in animals) (Petrick et al. 2001) and *in vitro* (human cell lines) (Styblo et al. 2002). Several mechanisms by which arsenical compounds induce tumorigenesis have been proposed, including oxidative stress (Kitchin and Wallace 2008), genotoxic damage and chromosomal abnormalities (Moore et al. 1997a; Zhang et al. 2007a), and cocarcinogenesis with other environmental toxicants (Rossman et al. 2004); epigenetic mechanisms, in particular, have been reported to alter DNA methylation (Zhao et al. 1997).

It is generally believed that arsenic does not induce point mutations, based on negative findings in both bacterial and mammalian mutagenicity assays (Jacobson-Kram and Montalbano 1985; Jongen et al. 1985). Arsenic does induce deletion mutations, but arsenical compounds vary in their potency (Moore et al. 1997b). With respect to arsenic's ability to induce chromosomal alterations in humans, studies in the early 1990s showed that the cell micronucleus assay could be used as a biological marker of the genotoxic effects of arsenic exposure (Smith et al. 1993). Later studies validated this assay and demonstrated higher frequencies of micronuclei in individuals who were chronically exposed to arsenicals (Moore et al. 1997a). Analysis of chromosomal alterations in DNA from bladder tumors of 123 patients who had been exposed to arsenic in drinking water showed that tumors from patients with higher estimated levels of arsenic exposure had higher levels of chromosomal instability than did tumors from patients with lower estimated levels of exposure, suggesting that bladder tumors from arsenic-exposed patients may behave more aggressively than do tumors from unexposed patients (Moore et al. 2002). Based on these overall findings, a plausible and generally accepted mechanism for arsenic carcinogenicity is the induction of structural and numerical chromosomal abnormalities through indirect effects on DNA. However, as has been demonstrated for several tumors, including urothelial and hematological malignancies (Fournier et al. 2007; Muto et al. 2000), it is likely that interrelated genetic and epigenetic mechanisms together contribute to the toxicity and carcinogenicity of arsenic (Hei and Filipic 2004; Zhao et al. 1997).

Epigenetic Modifications Induced by Arsenic

Epigenetic alteration, which is not a genotoxic effect, leads to heritable phenomena that regulate gene expression without involving changes in the DNA sequence (Feinberg and Tycko 2004) and thus could be considered a form of potentially reversible DNA modification. Recent mechanistic studies of arsenic carcinogenesis have directly or indirectly shown the potential involvement of altered epigenetic regulation in gene expression changes induced by arsenic exposure. We recently showed that urinary defensin, beta 1 (DEFB1) protein levels were significantly decreased among men highly exposed to arsenic in studies conducted

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in Nevada (USA) and in Chile (Hegedus et al. 2008). DNA methylation is thought to play a role in regulating *DEFB1* expression (Sun et al. 2006). Follow-up studies are under way in our laboratory to determine if reduced levels of DEFB1 in exposed populations are due to arsenic-induced targeted gene silencing. Several studies have observed extensive changes in global gene expression in individuals after arsenic exposure (Andrew et al. 2008; Bailey et al. 2009; Bourdonnay et al. 2009; Xie et al. 2007). Further, maternal exposure to arsenic has been shown to alter expression of transcripts in the mouse fetus (Liu et al. 2008) and human newborn (Fry et al. 2007). Because epigenetic processes are major regulators of gene expression, these findings suggest that dysregulation of epigenetic processes could contribute mechanistically to arsenic-induced changes in gene expression and cancer, affecting both people exposed to arsenic directly and those of future generations in a heritable manner, without directly altering the genome. Dysregulation of epigenetic processes could also contribute to vascular disease (Yan et al. 2010) and neurological disorders (Urdinguio et al. 2009).

Many groups have directly examined the association of arsenic exposure on epigenetic phenomena; because the technologies used to study the various epigenetic modifications are developing rapidly, we believe that a review of current findings from the literature is warranted. We conducted a PubMed search (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) through April 2010 and identified studies using variable keywords, such as "arsenic AND DNA methylation," "arsenic AND microRNA," "arsenic AND histone modification," and "arsenic AND epigentics AND epigenomics." Our goal was to include all the studies we could find, and thus the reference lists of the identified studies were also reviewed to identify other relevant studies. Although epigenetic alterations may contribute to effects of arsenic on both cancer and noncancer outcomes, in this article we summarize the recent *in vitro* and *in vivo* research findings on the potential role of arsenic-mediated epigenetic alterations in arsenic-induced toxicity and carcinogenicity. We discuss three major epigenetic mechanisms proposed to play roles in arsenic-induced carcinogenesis: altered DNA methylation, histone modification, and microRNA (miRNA) expression. We also propose future directions that can further inform our understanding of the epigenetic and overall mechanisms underlying the effects of arsenic.

Arsenic Exposure and DNA Methylation

DNA methylation is tightly regulated in mammalian development and is essential for maintaining the normal functioning of the adult organism (Schaefer et al. 2007). Altered DNA methylation has been associated with several human diseases (Robertson 2005). Global genomic DNA hypomethylation is a hallmark of many types of cancers (Esteller et al. 2001), resulting in illegitimate recombination events and causing transcriptional deregulation of affected genes (Robertson 2005). In mammalian systems, DNA methylation occurs predominantly in cytosine-rich gene regions, known as CpG islands, and serves to regulate gene expression and maintain genome stability (Yoder et al.

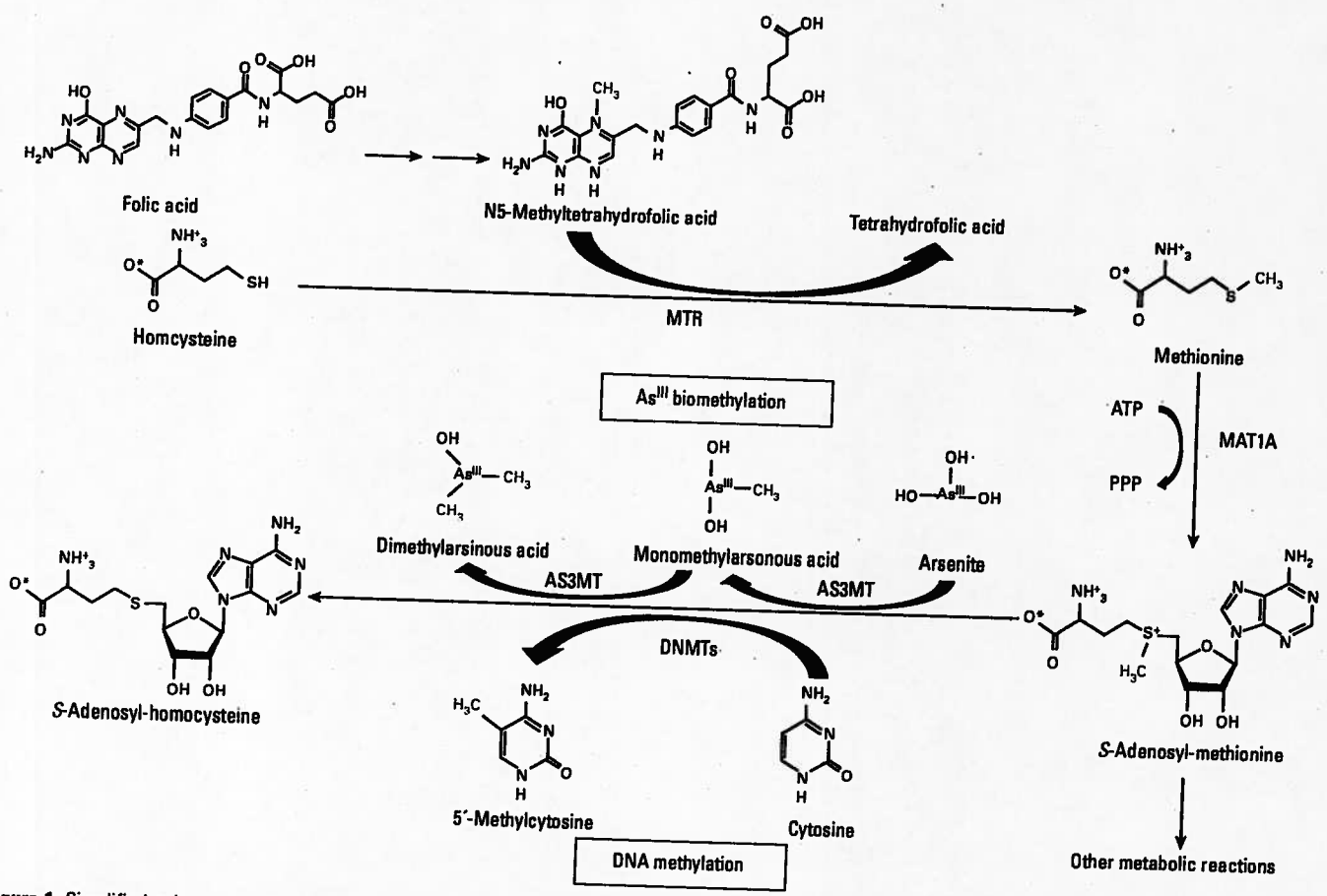

Figure 1. Simplified scheme of SAM synthesis and its involvement in arsenic and DNA methylation. The human arsenic metabolic pathway involves a series of methylation reactions; both arsenic metabolism and DNA methylation require SAM as the methyl donor. Here we show the intermediate steps of SAM synthesis and its involvement in the methylation of DNA and arsenic. Abbreviations: AS3MT, arsenic (+3 oxidation state) methyltransferase; ATP, Adenosine-5'-triphosphate; MAT1A, methionine adenosyltransferase I; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; PPP, tripolyphosphate.

1997). DNA methyltransferases (DNMTs) are responsible for transferring a methyl group from the *S*-adenosyl methionine (SAM) cofactor to the cytosine nucleotide, producing 5′-methylcytosine and *S*-adenosyl homocysteine (Figure 1) (Razin and Riggs 1980). Three different families of *DNMT* genes have been identified so far: *DNMT1*, *DNMT2*, and *DNMT3* (Robertson and Wolffe 2000).

Mechanisms of arsenic-induced changes in DNA methylation. An association between arsenic-induced carcinogenesis and DNA methylation was proposed because arsenic methylation and DNA methylation both use the same methyl donor, SAM (Figure 1). SAM is a coenzyme involved in > 40 metabolic reactions that require methyl group transfers (Chiang et al. 1996; Loenen 2006; Reichard et al. 2007). Because SAM is the unique methyl group donor in each conversion step of biomethylation of arsenic, long-term exposure to arsenic may lead to SAM insufficiency and global DNA hypomethylation (Coppin et al. 2008; Goering et al. 1999; Zhao et al. 1997). Further, because SAM synthesis requires methionine, an essential amino acid in humans, dietary methyl insufficiency could exacerbate effects of arsenic on DNA methylation (Figure 1) (McCabe and Caudill 2005). Indeed, human exposure to arsenic often occurs in relatively resource-poor populations in developing countries that also may have low dietary intakes of methionine (Anetor et al. 2007). In addition to its effect on SAM availability, arsenic can directly interact with DNMTs and inhibit their activities. Several studies have shown that arsenic exposure leads to a dose-dependent reduction of mRNA levels and activity of DNMTs both *in vitro* and *in vivo*, including *DNMT1*, *DNMT3A*, and *DNMT3B* (Ahlborn et al. 2008; Cui et al. 2006b; Fu et al. 2007; Reichard et al. 2007).

Arsenic and global DNA hypomethylation. Global DNA hypomethylation is expected to result from arsenic exposure through both SAM insufficiency and reduction of *DNMT* gene expression (Reichard et al. 2007). Arsenic exposure has been reported to induce DNA hypomethylation *in vitro* and in animal studies (Table 1). For example, rats (Uthus and Davis 2005) and mice (Chen et al. 2004; Okoji et al. 2002; Xie et al. 2004) exposed to As^{III} for several weeks displayed global hepatic DNA hypomethylation. Similarly, exposure of fish to As^{III} for 1, 4, or 7 days resulted in sustained DNA hypomethylation compared with non-exposed fish (Bagnyukova et al. 2007). Studies in cell lines *in vitro* yielded similar results, with a reduction in global genomic DNA methylation resulting from As^{III} exposure (Table 1) (Benbrahim-Tallaa et al. 2005; Coppin et al. 2008; Reichard et al. 2007; Sciandrello et al. 2004; Zhao et al. 1997). In contrast to the animal and *in vitro* findings, there are limited human population studies available. A cross-sectional study of 64 people reported by Majumdar et al. (2010) indicated that exposure to arsenic-contaminated water (250–500 μg/L) was associated with global DNA hypermethylation. However, the participants in the highest estimated exposure group (> 500 μg/L) had methylation levels that were comparable with those in the two lowest groups. The one possible reason for this inconsistency may be that the actual intake of arsenic into the body is different in the participants whose exposures were estimated based on the concentrations in their drinking water. In another well-designed nested case–control study, Pilsner et al. (2007) assessed the relationship between arsenic and DNA methylation in 294 participants and observed a positive association between urinary arsenic and DNA hypermethylation. Plasma folate level apparently has a significant effect on the level of DNA methylation because a dose–response relation was evident only among participants with adequate folate levels (≥ 9 nmol/L) when estimates were stratified according to plasma folate level after controling for other factors. In a separate but closely related nested case–control study, Pilsner et al. (2009) found that individuals with hypomethylation of peripheral blood leukocyte (PBL) DNA were 1.8 (95% confidence interval, 1.2–2.8) times more likely to have skin lesions 2 years later after adjusting for age, urinary arsenic, and other factors. Pilsner et al. (2009) speculated that

> Adequate folate may be permissive for an adaptive increase in genomic methylation of PBL DNA associated with [arsenic] exposure, and that individuals who are similarly exposed but in whom the increase in genomic DNA methylation does not occur (or cannot be sustained) are at elevated risk for skin lesions.

Further studies are required to determine if exposure to As^{III} has differential effects on the status of DNA methylation across tissues, cells, and species.

Arsenic and gene promoter methylation. Although the effects of arsenic exposure on global genomic DNA methylation remain unclear, DNA hypomethylation or hypermethylation of promoters of some genes has been reported in human skin cancer (Chanda et al. 2006) and bladder cancer (Chen et al. 2007; Marsit et al. 2006c) associated with arsenic exposure. It has also been observed

Table 1. Arsenic exposure and global DNA methylation.

Model	Arsenical	Dose	Time (weeks)	Global DNA methylation	References
Human cells					
Prostate epithelial cell line RWPE-1	As^{III}	5 μM	16	Hypo	Coppin et al. 2008
Prostate epithelial cell line RWPE-1	As^{III}	5 μM	29	Hypo	Benbrahim-Tallaa et al. 2005
HaCaT keratinocytes	As^{III}	0.2 μM	4	Hypo	Reichard et al. 2007
Animal cells					
TRL 1215 rat liver epithelial cell line	As^{III}	125–500 nM	18	Hypo	Zhao et al. 1997
V79-Cl3 Chinese hamster cells	As^{III}	10 μM	8	Hypo	Sciandrello et al. 2004
Animal studies					
Goldfish	As^{III}	200 μM	1	Hypo	Bagnyukova et al. 2007
Fisher 344 rat	As^{III}	50 μg/g body weight	12	Hypo	Uthus and Davis 2005
129/SvJ mice	As^{III}	45 ppm	49	Hypo	Chen et al. 2004
C3H mice	As^{III}	85 ppm	1.5	Hypo	Waalkes et al. 2004
C57BL/6J mice	As^{III}	2.6–14.6 μg/g body weight	18.5	Hypo	Okoji et al. 2002
Homozygous Tg.AC mice	As^{III}	150 ppm	17	Hypo	Xie et al. 2004
	As^{V}	200 ppm			
	MMA^{V}	1,500 ppm			
	DMA^{V}	1,200 ppm			
Human subjects					
	As^{III}	2–250 μg/L	NA	Hyper	Pilsner et al. 2007; Majumdar et al. 2010
	As^{III}	2–250 μg/L	NA	Hypo (in skin lesion patients)	Pilsner et al. 2009

Abbreviations: Hyper, hypermethylated; Hypo, hypomethylated; NA, not available. See text for additional information on human subjects.

in human cell lines (Chai et al. 2007; Fu and Shen 2005; Jensen et al. 2008; Mass and Wang 1997), animal cell lines (Chen et al. 2001, Takahashi et al. 2002), animals (Cui et al. 2006a; Okoji et al. 2002; Waalkes et al. 2004), and humans (Chanda et al. 2006; Chen et al. 2007; Marsit et al. 2006b; Zhang et al. 2007b) exposed to arsenic (Table 2). Although this gene-specific effect observed in these studies could be due to study bias, because researchers examined only a small group of genes, the similar methylation pattern repeatedly reported in the same genes after arsenic exposure might also suggest that arsenic could selectively target specific genes. However, little is known about how DNA methylation is targeted to specific regions (Jones and Baylin 2002). Hypo- and hypermethylation of genes could mediate carcinogenesis through up-regulation of oncogene expression or down-regulation of tumor suppressor genes, respectively. Both observations have been reported. Hypomethylation of the promoter region of oncogenic *Hras1* and an elevated *Hras1* mRNA level was demonstrated in mice treated with sodium arsenite (Okoji et al. 2002). Similar results on mRNA expression and promoter hypomethylation of *Hras1* and *c-myc* were also observed *in vitro* (Chen et al. 2001; Takahashi et al. 2002). The evidence has linked overexpression of *Esr1* (estrogen receptor 1) gene with estrogen-induced hepatocellular carcinoma in mice (Couse et al. 1997). Arsenic exposure leads to overexpression of the *Esr1* gene resulting from hypomethylation of its promoter region, indicating an association between overexpression of *Esr1* and arsenic hepatocarcinogenesis (Chen et al. 2004; Waalkes et al. 2004).

Dose-dependent hypermethylation at the promoter region of several tumor suppressor genes [e.g., *p15, p16, p53*, and death-associated protein kinase (*DAPK*)] was induced by arsenic exposure *in vitro* and *in vivo* (Boonchai et al. 2000; Chanda et al. 2006; Fu and Shen 2005; Mass and Wang 1997; Zhang et al. 2007b). In a population-based study of human bladder cancer in 351 patients, *RASSF1A* and *PRSS3* promoter hypermethylation was positively associated with toenail arsenic concentrations, and promoter hypermethylation in both genes also was associated with invasive (vs. noninvasive low grade) cancer (Marsit et al. 2006b). This outcome was recapitulated in arsenic-induced lung cancer in A/J mice, in which the arsenic exposure reduced the expression of *RASSF1A* resulting from hypermethylation of its promoter region and was associated with arsenic-induced lung carcinogenesis (Cui et al. 2006a). DAPK is a positive mediator of γ-interferon–induced programmed cell death and a tumor suppressor candidate. In a study of 38 patients with urothelial carcinoma, Chen et al. (2007) reported hypermethylation of *DAPK* in 13 of 17 tumors in patients living in arsenic-contaminated areas compared with 8 of 21 tumors from patients living in areas not contaminated with arsenic. This hypermethylation of *DAPK* was also observed in an *in vitro* study when immortalized human uroepithelial cells were exposed to arsenic (Chai et al. 2007). The increase of DNA hypermethylation of promoter in *p16* was observed in arseniasis patients compared with people with no history of arsenic exposure (Zhang et al 2007b). In another study Chanda et al. (2006) examined the methylation status of promoters in *p53* and *p16* in DNA extracted from peripheral lymphocytes and observed an increase of methylation in both *p53* and *p16* associated with an estimated arsenic exposure in a dose-dependent manner. However, this same study also showed that the subjects from the highest arsenic exposure group exhibited hypomethylation of both *p53* and *p16*. Chronic exposure to arsenic *in vitro* has been shown to induce malignant transformation in several human cell types (Benbrahim-Tallaa et al. 2005; Zhao et al. 1997) in which the alteration of DNA methylation level has been shown to be involved (Jensen et al. 2008, 2009a; Zhao et al. 1997).

Summary. Arsenic does not fall into the classic model of carcinogenesis because it is not efficient at inducing point mutations or initiating and promoting the development of tumors in experimental animals. One likely mechanism by which arsenicals operate is through the disruption of normal epigenetic control at specific loci, which may result in aberrant gene expression and cancer (Andrew et al. 2008; Xie et al. 2007). Although there is increasing evidence that arsenic exposure alters methylation levels in both global DNA and promoters of some genes, the current available studies are essentially descriptive and difficult to interpret because of the complexity of the study populations and limited information provided in the reports. Studies are needed that systematically investigate DNA methylation on a genome-wide level in arsenic-exposed cell lines and in target tissues, such as exfoliated bladder cells, from well-characterized arsenic-exposed human populations, or in tumor tissue from arsenic-associated cancers. Such studies would help to clarify potential effects of arsenic exposure on DNA methylation and carcinogenesis.

Table 2. Arsenic exposure and gene-specific (promoter) methylation status.

Mode	Arsenical	Dose	Time (weeks)	Genes		Reference
				Hyper	Hypo	
Human cells						
UROtsa urothelial cells	As^{III}	1 μM	9	*DBC1, FAM83A, ZSCAN12, C1QTNF6*		Jensen et al. 2008
	MMA^{III}	50 nM				
Uroepithelial SV-HUC-1 cells	As^{III}	2, 4, 10 μM	24 or 52	*DAPK*		Chai et al. 2007
Myeloma cell line U266	As^{III}	1, 2 μM	0.4	*P16*		Fu and Shen 2005
Lung adenocarcinoma A549 cells	As^{III}	0.08–2 μM	0.3	*P53*		Mass and Wang 1997
	As^{V}	30–300 μM	0.3			
Animal cells						
Syrian hamster embryo cells	As^{III}	3–10 μM	0.3		c-*myc*, c-*Ha-ras*	Takahashi et al. 2002
	As^{V}	50–150 μM	0.3			
TRL 1215 rat liver epithelial cells	As^{III}	125–500 nM	8 or 18		c-*myc*	Chen et al. 2001
Animal studies						
C57BL/6J mice	As^{III}	2.6–14.6 μg/g body weight	18.5		c-*Ha-ras*	Okoji et al. 2002
A/J mice	As^{V}	100 ppm	74	*p16, RASSF1*		Cui et al. 2006a
C3H mice	As^{III}	85 ppm	1.4		*ERα*	Waalkes et al. 2004
Human subjects						
	As^{III}	NA	NA	*DAPK*		Chen et al. 2007
	As^{III}	Variable[a]	NA	*p53, P16*		Chanda et al. 2006
	As^{III}	NA	NA	*p16*		Zhang et al. 2007b
	As^{III}	Variable[b]	NA	*RASSF1A, PRSS3*		Marsit et al. 2006b

Abbreviations: ERα, estrogen receptor α; Hyper, hypermethylated; Hypo, hypomethylated; NA, not available.
[a]Study subjects were grouped based on historical arsenic concentration in drinking water, and the range of arsenic concentration in drinking water was < 50 μg/L to > 300 μg/L. [b]The estimated toenail arsenic concentration of study subjects was < 0.01 μg/L to > 50 μg/L.

Arsenic Exposure and Histone Modification

Chromatin is structured within the cell nucleus in units called nucleosomes, in which DNA is packaged within the cell. The nucleosome core particle consists of stretches of DNA (~ 146 bp) wrapped in left-handed superhelical turns around a histone octamer consisting of two copies each of the core histones H2A, H2B, H3, and H4 (Luger et al. 1997). Although H1 does not make up the nucleosome "bead," H1 plays a role in keeping in place the DNA that has wrapped around the nucleosome (Figure 2). From a structural and functional perspective, histones have different characteristics depending on the number of amino acids and the number and type of covalent modifications in these residues. These covalent modifications, found in the tails of the histone chains, influence many fundamental biological processes including acetylation, methylation, phosphorylation, citrullination, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization (Kouzarides 2007) (Figure 2). To date, published studies on histone modifications and arsenic toxicity have focused on acetylation, methylation, and phosphorylation.

Histone acetylation. Histone acetylation is a dynamic and reversible event (Glozak and Seto 2007), in which the acetylation status of lysine residues in the histone tail is regulated by two antagonistic enzyme classes, histone acetyltransferases (HATs) (Sterner and Berger 2000) and histone deacetylases (HDACs) (Cress and Seto 2000). Using acetyl coenzyme A as an acetyl group donor, HATs enzymatically transfer a single acetyl group to the ε-amino group of specific lysine side chains within the histone's basic N-terminal tail region, whereas HDACs remove the acetyl group from the lysine residues.

Evidence for an association between altered histone acetylation and arsenic-induced toxicity continues to be strengthened. In the early 1980s, arsenic exposure was shown to significantly reduce histone acetylation in *Drosophila* (Arrigo 1983). More recently, changes in histone H3 acetylation have been observed in association with As^{III}- and MMA^{III}-induced malignant transformation of human urothelial cells *in vitro*; these modifications apparently are arsenic specific because the co-occurring changes in both As^{III}- and MMA^{III}-induced malignant transformation are significantly more frequent than those occurring by random chance (Jensen et al. 2008). Further, Jensen et al. (2008) reported DNA hypermethylation in a number of the hypoacetylated promoters identified in the study, suggesting that arsenic coordinately targets genes through dysregulation of different epigenetic mechanisms contributing to malignant transformation. Recently, we showed that the global level of H4K16 acetylation in human bladder epithelial cells was reduced in a dose- and time-dependent manner by both As^{III} and MMA^{III} treatment (Jo et al. 2009). Moreover, knockdown of *MYST1*, the gene responsible for H4K16 acetylation, resulted in increased cytotoxicity from arsenical exposure in human bladder epithelial cells, suggesting that H4K16 acetylation may be important for resistance to arsenic-induced toxicity.

Interestingly, As^{III} exposure has also been shown to induce elevated histone acetylation, which was reportedly responsible for the up-regulation of genes involved in apoptosis or the response to cell stress after exposure to arsenic (Li et al. 2002, 2003). This result probably is mediated by HDACs. As^{III} has been shown to inhibit *HDAC* genes that correlate with increased global histone acetylation (Ramirez et al. 2008). The level of inhibition is comparable with that of the well-known HDAC inhibitor trichostatin A (Drummond et al. 2005). Together, these studies clearly provide evidence that histone acetylation is dysregulated by arsenic exposure, but further work is needed to understand the underlying mechanisms and to clarify the net effect of altered histone acetylation on arsenic-induced toxicity and carcinogenesis.

Histone methylation. Like acetylation, histone methylation is also a reversible process. However, unlike acetylation, which occurs only on lysine residues at the histone tail, histone methylation occurs on both lysine and arginine residues (Martin and Zhang 2005; Wysocka et al. 2006). In mammals, histone methylation is usually found on histone H3 and H4, although it also occurs on H2A or H2B. Arginine methylation is catalyzed by the enzyme arginine *N*-methyltransferase (Wysocka et al. 2006), whereas lysine methylation is catalyzed by two different classes of proteins, the SET-domain–containing protein family and the non-SET-domain proteins DOT1/DOT1L (Martin and Zhang 2005). Histone methylation can occur in the monomethyl, symmetrical dimethyl, and asymmetrical dimethyl states and in the trimethyl group states, in contrast to the single acetyl group added during acetylation (Klose and Zhang 2007). Histone methylation was considered a static modification until recent years, when enzymes were found to be capable of antagonizing histone arginine methylation or directly removing a methyl group from a lysine residue of histone (Klose and Zhang 2007). These enzymes include peptidylarginine deiminase enzymes and amine oxidase– and JmjC domain–containing histone demethylase enzymes.

Accumulating evidence implicates the aberrant loss or gain of histone methylation in tumorigenesis (Schneider et al. 2002). Arrigo (1983) first reported that exposure to arsenic in *Drosophila* cells led to a complete abolishment of methylation of histones H3 and H4, and the effect on H3 was later confirmed by other investigators (Desrosiers and Tanguay 1986, 1988). The response to arsenic exposure in the mammalian cell is more complex, and As^{III} treatment can lead to differential effects on the methylation of H3 lysine residues, including increased H3 lysine 9 dimethylation (H3K9me2) and H3 lysine 4 trimethylation (H3K4me3) and decreased H3 lysine 27 trimethylation (H3K27me3) (Zhou et al. 2008). Zhou et al. (2009) showed that 1 μM arsenite

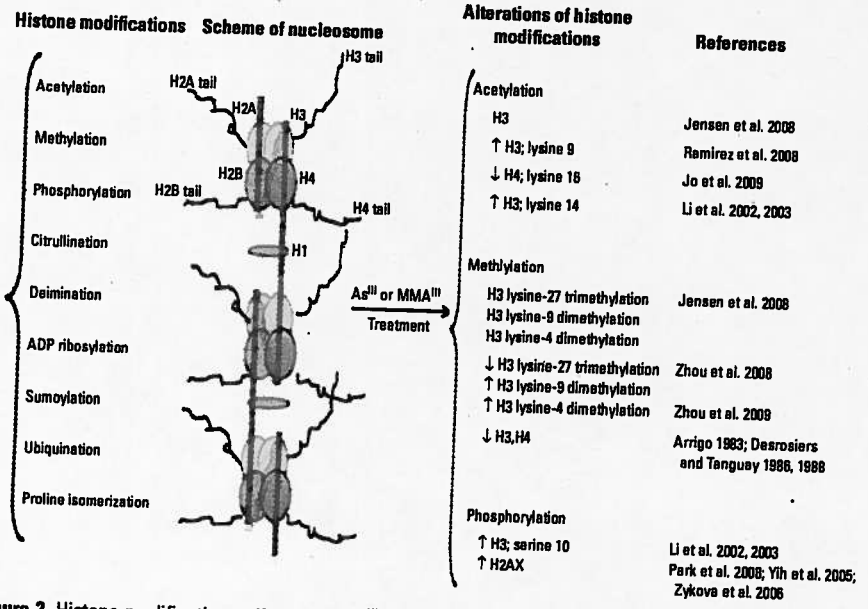

Figure 2. Histone modifications affected by As^{III} and MMA^{III} exposure. Major posttranscriptional histone modifications of the nucleosome are listed on the left. Modifications of specific histone proteins reported in the literature as altered by arsenic exposure are shown on the right.

significantly increased H3K4me3 after 24-hr or 7-day exposures in human lung carcinoma A549 cells. Importantly, H3K4me3 remained elevated, apparently inherited through cell division, 7 days after the removal of arsenite. Elevated H3K9me2, mediated by increased levels of histone methyltransferase G9a protein (Zhou et al. 2008), correlates with transcriptional repression (Peterson and Laniel 2004) and has been shown to be involved in the silencing of tumor suppressers in the cancer cell lines (Esteve et al. 2007; McGarvey et al. 2006). However, data on the patterns of histone methylation induced by arsenic exposure are limited, and further studies are required to decipher the relationship between altered histone methylation and gene expression, as well as its effect on arsenic-induced carcinogenesis.

Histone phosphorylation. All four core histone proteins, H2A, H2B, H3, and H4, and the linker histone H1 can be post-translationally modified by phosphorylation. Cyclin-dependent kinases are believed to be responsible for H1 phosphorylation (Swank et al. 1997). Several kinases are able to phosphorylate H2A and H2B, such as ataxia telangiectasia mutated for H2AX (Burma et al. 2001). Phosphorylation of H3 has been specifically implicated in cell cycle progression and regulation of gene expression (Houben et al. 2007). Similarly, phosphorylation of histone H4 (serine 1) increases during the cell cycle and is believed to be regulated by casein kinase 2 (Barber et al. 2004).

Histone phosphorylation may also contribute to arsenic-induced carcinogenesis. Although all four core histones (H2A, H2B, H3, and H4) are targets of protein kinases (Peterson and Laniel 2004), the best-studied histone phosphorylation event is that of H2AX, a form of H2A that represents up to 25% of the total H2A pool in mammals. Zykova et al. (2006) demonstrated that arsenic trioxide induces apoptosis by up-regulation of phosphorylated H2AX and may be one of the mechanisms by which arsenic trioxide acts as an antineoplastic agent (Figure 2). Little is known about histone phosphorylation and arsenic carcinogenesis. Studies have suggested that H3 phosphorylation induced by arsenic exposure might be responsible for the up-regulation of the oncogenes c-*fos* and c-*jun* (Li et al. 2003) and induction of a protoapoptotic factor, caspase 10 (Li et al. 2002). Nickel, another important metal with epigenetic effects, has been shown to induce phosphorylation of histone 3, specifically H3S10 (serine 10) via the activation of the JNK/SAPK (c-jun N-terminal kinase/stress-activated protein kinase) pathway (Ke et al. 2008). Because arsenite exposure is known to activate JNK and p38/Mpk2 kinase by inhibition of the corresponding protein phosphatases (Cavigelli et al. 1996), phosphorylation of histone H3 via the JNK/SAPK pathway might be a common mechanism of metal-induced histone modification.

Different types of histone modifications have been shown to affect gene regulation and expression in a coordinated manner. For example, *WNT5A* gene expression is up-regulated in As^{III}- and MMA^{III}-induced malignant transformation in uroepithelial cells in association with the enrichment of permissive histone modifications and reduction of repressive modifications in the *WNT5A* promoter region (Jensen et al. 2009b). Two modifications of histone H3, dimethylation of H3K4 and acetylation of H3K9 and H3K14, are associated with transcriptional competency, whereas the other two modifications of histone H3, trimethylation of H3K27 and dimethylation of H3K9, are correlated with transcriptional repression (Peterson and Laniel 2004).

Summary. Although we are still in the early stages of elucidating the association between histone modifications induced by arsenic and their effects on arsenic carcinogenicity, newly available techniques such as mass spectrometry (MS)-based histone modification analysis and genomewide sequencing offer the potential to systematically characterize the altered histone modifications induced by arsenicals and the subsequent changes in gene expression.

Arsenic Exposure and miRNA Expression

In the past few years, several laboratories have discovered a small class of non-protein-coding RNAs, called microRNAs (miRNAs), that participate in diverse biological regulatory events and are transcribed mainly from non-protein-coding regions of the genome (Bartel 2004; He and Hannon 2004). More than 700 human miRNAs have been identified to date, as documented in the miRBase database (Release 14; miRBase 2009), and it is predicted that many more exist. Each miRNA is thought to target several hundred genes, and as many as 30% of mammalian genes are regulated by miRNA (Lewis et al. 2005). miRNAs deactivate gene expression by binding to the 3′-untranslated region of mRNA with incomplete base pairing (Wightman et al. 1993). The exact mechanisms by which expression is repressed are still under investigation but may include the inhibition of protein synthesis, the degradation of target mRNAs, and the translocation of target mRNAs into cytoplasmic processing bodies (Jackson and Standart 2007). Because of the suppressive effect of miRNA on gene expression, a reduction or elimination of miRNAs that target oncogenes could result in the inappropriate expression of those oncoproteins; for example, Johnson et al. (2005) have shown that *RAS* oncogene is regulated by the let-7 miRNA family. Conversely, the amplification or overexpression of miRNAs that have a role in regulating the expression of tumor suppressor genes could reduce the expression of such genes. A prime example of this is the observation of the miR-34 family on the p53 tumor suppressor pathway (He et al. 2007).

Altered miRNA expression and arsenic exposure. Despite the significant progress made toward understanding the biogenesis and mechanisms of action of miRNA, much less is known about the effect of environmental exposures, especially carcinogens such as arsenic, on miRNA expression. Several studies have shown that exposure to exogenous chemicals can alter miRNA expression (Kasashima et al. 2004; Pogribny et al. 2007; Shah et al. 2007). *In vitro* exposure of cells to iron sulfate or aluminum sulfate, which generate reactive oxygen species (ROS), led to the up-regulation of a specific set of miRNAs, including miR-9, miR-125b, and miR-128 (Lukiw and Pogue 2007). ROS generation resulting from arsenic exposure is thought to play a large role in arsenic-induced carcinogenesis and toxicity (Flora et al. 2007; Hei and Filipic 2004) and could potentially alter these miRNAs in a similar manner. Marsit et al. (2006a) examined the roles that arsenic and folate deficiency play in miRNA expression; these authors found that human lymphoblast TK6 cells that had been treated with sodium arsenite and cells that had been grown in folate-deficient media over a 6-day period showed similarly altered expression of five miRNAs compared with untreated controls, suggesting a common mechanism of dysregulation. One such potential mechanism is aberrant DNA methylation occurring as a result of SAM depletion (Caudill et al. 2001; Loenen 2006), which arises under conditions of arsenic exposure and folate deficiency. However, Caudill et al. (2001) found no significant decrease in global methylation in the treated compared with the control groups, suggesting more subtle or targeted effects. The induced changes in miRNA expression were not stable and returned to baseline levels upon removal of the stress conditions, suggesting that chronic exposure may be necessary to permanently alter expression of miRNAs (Marsit et al. 2006a). Arsenic trioxide, a treatment option for acute promyelocytic leukemia (APL) (Zhou et al. 2005), induces the relocalization and degradation of the nuclear body protein promyelocytic leukemia (PML) protein, as well as the degradation of PML–retinoic acid receptor-α (PML-RARα) in APL cells (Shao et al. 1998). APL patients treated with all-*trans* retinoic acid release a group of miRNAs transcriptionally repressed by the APL-associated PML-RAR oncogene (Saumet et al. 2009), suggesting that arsenicals may produce similar effects on miRNA expression in APL patients.

Summary. Overall, these studies show that environmental carcinogen exposures can lead to altered miRNA expression profiles,

which may be associated with the process of carcinogenesis. Further studies are necessary to clarify whether chronic exposure to arsenic is capable of altering miRNA expression and what biological effects are related to the altered miRNA expression.

Epigenomic Approach Proposed for Future Studies

Emerging evidence suggests that arsenic acts through several epigenetic mechanisms. The characterization of genomewide patterns of DNA methylation, posttranslational histone modification, and miRNA expression after arsenic exposure *in vitro* and *in vivo* represents a new frontier toward our understanding of the mechanisms of arsenic toxicity and carcinogenesis. Emerging epigenomic technologies such as chromatin immunoprecipitation (ChIP)-on-chip and ChIP sequencing (ChIP-seq), global methylation, and miRNA microarrays, as well as whole genome DNA sequencing platforms, will facilitate these efforts (Schones and Zhao 2008). ChIP-on-chip and ChIP-seq, used primarily to determine how proteins interact with DNA, have the potential to clarify how epigenetic changes, particularly histone modifications, induced by arsenic exposure regulate gene expression (Park 2009). MS offers an unbiased approach to mapping the combinations of histone modifications and requires highly sensitive and precise mass measurements; for example, the difference in mass between trimethylation and acetylation is only 36 mDa. Using liquid chromatography–MS, we identified acetylation of H4K16 as a histone modification that is significantly reduced after arsenic treatment, especially with long-term exposure (Jo et al. 2009).

With the rapid development of array and sequencing-based DNA-methylation profiling technologies, global DNA methylation profiling has clearly come of age. Because epigenetic modifications alter gene expression but not gene sequence, transcriptomics may eventually allow the characterization of the expression profiles of epigenetically labile genes. Identification of the genes dysregulated through epigenetic mechanisms by arsenic exposure will further elucidate the associated biological processes and disease states. Proteomics using both conventional "bottom-up" and newer cutting-edge "top-down" MS approaches to detect labile posttranslational modifications that are often lost in conventional MS/MS experiments will allow further clarification of the resulting phenotype. The difference between these two approaches is that the materials introduced into the mass spectrometer are either peptides generated by enzymatic cleavage of one or many proteins in the "bottom-up" approach, or intact protein ions or large protein fragments in the "top-down" approach. Integration of epigenetic, transcriptomic, and proteomic data sets generated by these techniques will facilitate a more thorough understanding of the interplay of these processes under normal conditions and during arsenic exposure. Indeed, the importance of a comprehensive understanding of the epigenome has been recognized by the scientific community and is reflected in the National Institutes of Health (NIH) Roadmap Initiative (NIH 2007) with the goal of developing comprehensive reference epigenome maps and new technologies for comprehensive epigenomic analyses.

Conclusion and Future Directions

Although experiments in suitable model systems could complement the human studies, as discussed above, there may be differences between epigenetic effects in animals and humans and between various tissues and cell types. Thus, studies in human populations exposed to high levels of arsenic will be necessary to understand how individual differences in arsenic methylation and genetic background, as well as environmental factors such as diet and age, influence the epigenetic response to chronic arsenic exposure. Studies will also be required across various tissue and cell types to identify and validate the levels and patterns of epigenetic markers in these cells. Accessible tissues such as blood may not represent a good surrogate of target tissues such as bladder, kidney, and lung. High-resolution methylation data have shown that tissues have distinct epigenetic profiles (Christensen et al. 2009; Illingworth et al. 2008), and aging and environmental exposures may alter methylation in a tissue-specific manner (Christensen et al. 2009). Thus, epigenetic profiles from disease-relevant tissues such as exfoliated bladder cells from exposed and unexposed disease-free individuals could allow early effects to be identified. Such cells could also be analyzed from individuals with arsenic- and non–arsenic-associated cancers to identify arsenic-associated tumorigenic profiles. Rosser et al. (2009) showed that it may be possible to detect bladder cancer using gene expression signatures in exfoliated bladder urothelia. Similarly, the effects of inhaled arsenic on epigenetic profiles in bronchial airway epithelial cells could be examined in exposed and unexposed disease-free individuals and those with lung cancer, as was recently done using miRNA profiling for cigarette smoke exposure (Schembri et al. 2009).

In conclusion, a comprehensive epigenomic approach may elucidate the mechanisms of arsenic-induced carcinogenesis. Such an approach would also facilitate the discovery of biomarkers of arsenic exposure and early effects, associated diseases and disease progression, and factors that confer susceptibility.

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Epigenetic remodeling during arsenical-induced malignant transformation

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Humans are exposed to arsenicals through many routes with the most common being in drinking water. Exposure to arsenic has been associated with an increase in the incidence of cancer of the skin, lung and bladder. Although the relationship between exposure and carcinogenesis is well documented, the mechanisms by which arsenic participates in tumorigenesis are not fully elucidated. We evaluated the potential epigenetic component of arsenical action by assessing the histone acetylation state of 13 000 human gene promoters in a cell line model of arsenical-mediated malignant transformation. We show changes in histone H3 acetylation occur during arsenical-induced malignant transformation that are linked to the expression state of the associated gene. DNA hypermethylation was detected in hypoacetylated promoters in the select cases analyzed. These epigenetic changes occurred frequently in the same promoters whether the selection was performed with arsenite [As(III)] or with monomethylarsonous acid, suggesting that these promoters were targeted in a non-random fashion, and probably occur in regions important in arsenical-induced malignant transformation. Taken together, these data suggest that arsenicals may participate in tumorigenesis by altering the epigenetic terrain of select genes.

Introduction

Humans are exposed to many forms of environmental arsenic with the inorganic arsenicals, arsenate [As(V)] and arsenite [As(III)], being the most abundant forms. Human exposure to inorganic arsenic occurs most commonly through contaminated drinking water. Once ingested, inorganic arsenic is enzymatically biotransformed to a number of metabolites including both trivalent and pentavalent methylated species (1,2). The methylated trivalent arsenicals are highly toxic forms of arsenic and have been detected in human urine with monomethylarsonous acid [MMA(III)] being of particular interest (3–5). MMA(III) is a more potent toxicant than arsenite, with its cytotoxic potency being 20 times that of the parent compound (6,7). Epidemiological studies have indicated that exposure to arsenicals leads to a multitude of health problems including cancers of the lung, skin and bladder (8–11). A recent report detailed a 50 years epidemiological study that examined the incidence of bladder cancer relative to arsenic exposure in Chilean populations. Results suggested that a significant increase in bladder cancer mortality, peaking with a rate ratio of 6.1:13.8, was linked to higher levels of arsenic in the drinking water (10).

Although arsenic is classified as a human carcinogen, the mechanisms by which arsenicals lead to the formation of neoplasms remain unclear and are probably multifactorial. Previous studies have shown that arsenicals can alter growth signaling pathways (12,13). Arsenicals are classified as weak mutagens by most classical mutagenesis assays; however, they can indirectly damage DNA via the generation of reactive oxygen species and inhibition of DNA replication and repair enzymes (14–20). These mechanisms clearly play a role in arsenical-mediated effects on cells; however, the long-term effects of arsenicals on cellular phenotype may be mediated through additional mechanisms as well.

Cells may adapt to long-term arsenical exposure through epigenetic mechanisms of gene control. Molecular toxicological studies on the heavy metals nickel and arsenic support this possibility. Using various cell types, it has been demonstrated that exposure to nickel can result in an altered chromatin state, specifically a decrease in histone acetylation (21–23). In addition, it has been shown, both *in vitro* and *in vivo*, that arsenic exposure results in the loss of genomic DNA methylation in rat hepatocytes (24,25). This relationship between the DNA methylation status of select promoters and arsenic exposure is also observed in human bladder cancer specimens (26). These studies shed light upon gene–environment interactions that involve the epigenetic control of carcinogenesis. Currently, however, little is known about the histone modification profile that results from chronic, low-level exposure to As(III) and MMA(III).

In light of recent research suggesting that environmental agents can perturb the epigenetic terrain, we initiated studies to address the role of arsenicals on the epigenome during malignant transformation. We used an immortalized, non-tumorigenic cell line model of human urothelial cells, UROtsa, and malignantly transformed variants of UROtsa that emerged from long-term exposure to arsenicals (27,28). These cells were malignantly transformed through chronic exposure to either 1 μM As(III) or 50 nM MMA(III), resulting in two independent cell line derivatives termed URO-ASSC and URO-MSC, respectively (7,29). Initial studies showed chronic treatment of UROtsa cells with As(III) resulted in the generation of cells showing cancer phenotypes including hyperproliferation, anchorage-independent growth and tumor formation after heterotransplantation into nude mice (29). Similar findings were independently obtained by Bredfeldt *et al.* after exposing UROtsa cells to 50 nM MMA(III). After 24 weeks of treatment (URO-MSC24), cells showed anchorage-independent growth and hyperproliferation but did not form tumors when injected into severe combined immunodeficiency mice. After 52 weeks of MMA(III) treatment, these cells (URO-MSC52) acquired the ability to form tumors in SCID mice in addition to exhibiting anchorage-independent growth (7). These UROtsa cell lines provide an unique model in which to study the epigenomic events that occur during arsenical-mediated tumorigenesis.

To study the epigenetic effects that occur during As(III)- and MMA(III)-induced malignant transformation, we employed an epigenomic scanning approach using a human promoter microarray platform. In this study, we show that the transformation of UROtsa cells with both As(III) and MMA(III) is associated with changes in histone H3 acetylation patterns. Additionally, the promoters that lost this permissive histone modification could be associated with an increase in DNA methylation in these regions and a repression of gene expression. Conversely, promoters that showed an increase in histone acetylation also revealed an increase in gene expression. Thus, these multilayered epigenetic changes probably play a functional role since the expression of the associated genes is linked to the epigenetic landscape present in their promoter regions. Taken together, these data provide a genome-wide view of the epigenetic changes associated with chronic arsenical exposure and provide new gene targets that participate in arsenical-induced tumorigenesis.

Materials and methods

Cell culture

UROtsa and URO-ASSC cells were kindly provided by the laboratory of Donald and Mary Ann Sens. URO-MSC24 and URO-MSC52 cell lines were provided by the laboratory of A.J.G. All cells were cultured in Dulbecco's modified Eagle's medium (Cellgro, Herndon, VA) supplemented with 5% vol/vol fetal bovine serum (Omega Scientific, Tarzana, CA) and 1% vol/vol

Abbreviations: 5-aza-dCyd, 5-aza-2′-deoxycytidine; MeDIP, methylated DNA immunoprecipitation; MMA, monomethylarsonous acid; PCR, polymerase chain reaction; TSA, trichostatin A.

penicillin–streptomycin (Cellgro) and maintained in 150 cm^2 culture flasks (Greiner Bio-One, Monroe, NC) at 37°C with 5% CO_2 as described by Bredfeldt *et al.* (7).

Drug treatment of URO-ASSC cells

URO-ASSC cells were treated with 5-aza-2′-deoxycytidine (5-aza-dCyd), trichostatin A (TSA) or both of these drugs as described previously (30). For 5-aza-dCyd treatment, cells were treated with 10 μM 5-aza-dCyd (Sigma, St Louis, MO) for 96 total hours with media and 5-aza-dCyd replaced after 48 h. TSA treatments were performed using 300 nM TSA (Sigma) for 24 h. Combination treatment with both 5-aza-dCyd and TSA was performed following the procedure as described previously for 5-aza-dCyd treatment with TSA added for the final 24 h.

Chemicals

Sodium arsenite, phenylmethylsulphonyl fluoride, aprotinin and pepstatin A were obtained from Sigma–Aldrich (St Louis, MO). Diiodomethylarsine [MMA(III) iodide, CH_3AsI_2] was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar *et al.* (31).

Isolation of nucleic acids

Nucleic acids were isolated as described previously (32). All samples were quantified using absorbance at 260 nm using the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Chromatin immunoprecipitation

Chromatin immunoprecipitations (ChIPs) were performed as described previously (33). Briefly, cells were treated with 1% formaldehyde for 10 min to cross-link DNA and protein. Cells were then scraped from culture plates in Hank's balanced salt solution (Cellgro) containing the protease inhibitors phenylmethylsulphonyl fluoride, aprotinin and pepstatin A. Resulting DNA–protein complexes were sonicated and subjected to gel electrophoresis to ensure proper sonication. A portion of the product was removed for later analysis as input DNA. The remaining portion was precleared using protein A Sepharose GL-4B beads (GE Healthcare, Piscataway, NJ) and incubated overnight with an antibody directed toward a specific histone modification. Antibodies against acetylated histone H3 and trimethylated histone H3 lysine-27 were purchased from Upstate/Millipore (Billerica, MA). Antibodies against dimethylated histone H3 lysine-4 and dimethylated histone H3 lysine-9 were purchased from Abcam (Cambridge, MA). After incubation, the bound DNA was immunoprecipitated, washed and treated with 5 M NaCl to reverse DNA–protein cross-links, after which protein was digested with proteinase K (Fermentas, Glen Burnie, MD). Immunoprecipitated and input DNA samples were purified using the PCR Purification Kit (Qiagen, Valencia, CA) and were quantified using the Quant-IT Picogreen dsDNA detection kit (Invitrogen, Carlsbad, CA). Fluorescence was measured using the Biotek FLx800 microplate reader (Biotek, Winooski, VT).

Human promoter microarray

Primers for the human promoter microarray probes were obtained from the Whitehead Institute (Cambridge, MA) (34). Microarray probes were generated by adding 100 ng genomic DNA from normal, human mononuclear cells to 45 μl of PCR master mix (Eppendorf, Hamburg, Germany) with polymerase chain reaction (PCR) primers (20 pmoles each) added. PCRs were performed in a 96-well format (ABgene, Rochester, NY) using MJ thermal cyclers (MJ Research, Waltham, MA). After completion of PCR, a 3 μl aliquot of PCR product was analyzed by gel electrophoresis in a 96-well format using the Invitrogen 2% agarose E-Gel 96 system (Invitrogen). Remaining product was then purified using the QIAquick 96 PCR Purification Kit (Qiagen). After clean up, PCR products were quantified. DNA was then lyophilized and resuspended in 10 μl 3× sodium chloride-sodium citrate buffer for printing onto activated microarray slides (Corning, Lowell, MA) using the OmniGrid Robot (Gene Machines, San Carlos, CA).

Promoter microarray hybridization

Equal amounts of input and ChIP DNA (100 ng) were amplified using the BioPrime Array CGH Genomic Labeling Module (Invitrogen) according to the modified manufacturer's protocol. Resulting amplified DNA was purified using the PCR Purification Kit (Qiagen) and quantified, after which equal amounts of input and ChIP DNA (1 μg) were subjected to another round of amplification using the BioPrime Array CGH Genomic Labeling Module (Invitrogen), this time incorporating cyanine-labeled deoxyuridine triphosphate (GE Healthcare). Input DNA was labeled with cyanine-5, whereas the immunoprecipitated DNA was labeled with cyanine-3. Labeled DNA was again purified using the PCR Purification Kit (Qiagen) and quantified to ensure proper amplification and incorporation of labeled deoxyuridine triphosphate using the microarray function of the NanoDrop 1000 Spectrophotometer (NanoDrop). Input and ChIP DNA were then combined, to which was added human COT-I DNA (Invitrogen) and yeast transfer RNA (Invitrogen), and the resulting mix was lyophilized to dryness. Dried target was then resuspended in Domino Oligo Hybridization Buffer (Gel Company, San Francisco, CA), denatured and applied to the human promoter microarray slide. Slides were incubated for 16 h, washed and scanned using an Axon GenePix 4000B microarray scanner (Axon, Sunnyvale, CA).

ChIPs coupled to real-time PCR

Equal amounts of input and immunoprecipitated DNA (1 ng) were added to IQ supermix (Bio-Rad, Hercules, CA), promoter-specific primers and fluorescent probes (Roche, Basel, Switzerland) and were analyzed using the ABI 7500 Real-Time Detection System (Applied Biosystems, Foster City, CA). Values were calculated using the delta Ct method normalizing to the respective input for each sample. Primers were designed using Primer3 in conjunction with ProbeFinder version 2.40 software (Roche Applied Science, Basel, Switzerland). Statistics were calculated using an unpaired *t*-test between each transformed cell line and UROtsa. Primer sequences are available upon request.

Real-time reverse transcription–PCR

Total RNA (250 ng) was converted to complementary DNA according to the manufacturer's instructions (Applied Biosystems). Converted complementary DNA (10 ng) was added to IQ supermix (Bio-Rad), gene-specific primers and fluorescent probes (Roche) and subjected to real-time PCR analysis using Roche UniversalProbe technology (Roche) using the ABI 7500 Real-Time Detection System (Applied Biosystems). Results were calculated using the delta Ct method normalizing to β-actin expression for each sample. Primers were designed using Primer3 in conjunction with ProbeFinder version 2.40 software (Roche Applied Science). Statistics were calculated using an unpaired *t*-test between each transformed cell line and UROtsa. Primer sequences are available upon request.

Methylcytosine immunoprecipitation

RNase-treated genomic DNA (15 μg) was sonicated and analyzed using gel electrophoresis to ensure proper sonication. DNA was incubated overnight with an antibody to 5-methylcytosine (Aviva Systems Biology, San Diego, CA). Antibody–DNA complexes were then immunoprecipitated, washed and eluted using the QiaQuick Gel Extraction Kit (Qiagen). DNA was quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop). Real-time PCR for these samples was performed as described previously for histone H3 acetylation. Statistics were calculated using an unpaired *t*-test between each transformed cell line and UROtsa.

Sodium bisulfite sequencing

Sodium bisulfite sequencing was performed as described previously (35). Primers were designed using Methyl Primer Express v1.0 software (Applied Biosystems) to amplify a sequence located with the probe sequence located on the promoter microarray. For each cell line, 12 clones were sequenced with samples exhibiting <80% identity with the predicted sequence or <90% bisulfite conversion removed from analysis. Sequencing data were analyzed using the BiQ Analyzer (36). Primer sequences are available upon request.

Data analysis

All microarray data were processed in R programming environment (37). For normalization of all data, the Linear Models for Microarray Data (Limma) package was used (38). Differentially acetylated elements were identified using statistical approaches as described previously (39). To control for false discovery rate, a multiple testing correction was performed according to the methods described by Benjamini *et al.* (40).

Results

Histone H3 exhibits altered acetylation patterns in gene promoters

UROtsa cells are a non-tumorigenic cell line model of human urothelium. Independent exposures of UROtsa to As(III) and MMA(III) resulted in two distinct cell line models of arsenical-selected malignant transformation termed URO-ASSC and URO-MSC cell lines, respectively (Figure 1). We used these cell lines to examine whether there were consistent changes in the histone H3 acetylation state linked to arsenical-induced malignant transformation. In order to examine the effect of arsenical selection on histone modification state, we coupled ChIP to microarray hybridizations that probed 13 000 human gene promoters. A minimum of three independent experiments were analyzed using the promoter microarrays in order to confirm reproducibility and minimize the number of false positives. To

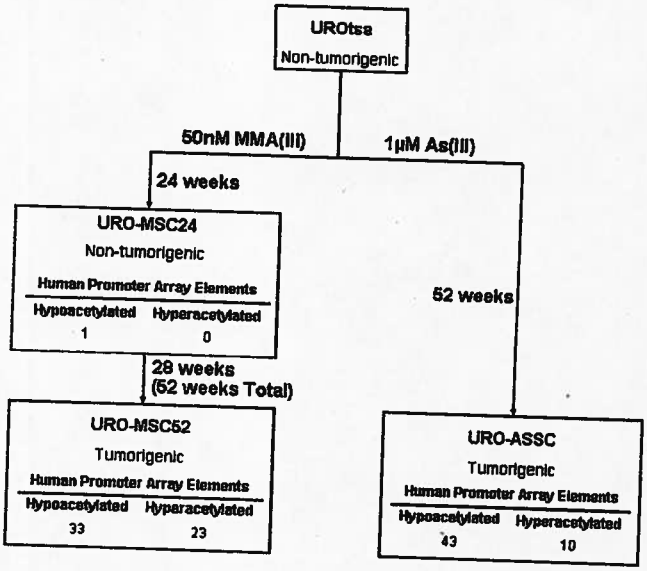

Fig. 1. Arsenical-induced malignant transformation results in histone H3 acetylation changes in gene promoters. UROtsa cells were exposed to As(III) (URO-ASSC) and MMA(III) (URO-MSC24, 52) with exposure time for each cell line described. Tumorigenicity classification is based on the ability of each cell line to form tumors when heterotransplanted into mice. ChIP experiments were coupled to human promoter microarrays in order to assess H3 acetylation state in UROtsa parent and the arsenical-exposed cell lines. Numbers shown represent the number of elements on the microarray showing significantly less (hypoacetylated) or significantly more (hyperacetylated) histone acetylation relative to the parental UROtsa cells (adjusted P-value < 0.05). Adjusted P-values were calculated according to the methods described by Benjamini *et al.* (40).

account for false discovery rate, P-values were adjusted according to the methods described by Benjamini *et al.* (40).

When compared with the UROtsa parental cell line, each transformed cell line showed changes in histone H3 acetylation in a select number of promoters (Figure 1). URO-MSC52 and URO-ASSC, the two cell lines able to form tumors when heterotransplanted into animals, displayed a similar number of promoters altered in a statistically significant fashion; 56 promoters in URO-MSC52 and 53 promoters in URO-ASSC (adjusted $P < 0.05$). When examining the promoter regions affected, it was striking that of the promoters affected in URO-MSC52 and URO-ASSC, 17 were altered in both cell lines. To determine if this number of changes could have co-occurred randomly, observed/expected ratios were calculated. If the changes were to have simply occurred as a result of random chance, one would expect that 0.26 elements from the microarray would be altered in both URO-ASSC and URO-MSC52 cell lines. The number of co-occurring changes observed (17) is 65 times higher than would have been expected by random chance, suggesting that the observed changes were arsenic specific.

The number of promoters altered in URO-MSC24 in relation to URO-MSC52 provides additional insights since it is an antecedent to URO-MSC52 and differs only by the duration of the chronic MMA exposure. In URO-MSC24, only one gene promoter showed a statistically significant change (decrease) in histone H3 acetylation, the promoter for *ZSCAN12*, a zinc finger protein from the Kruppel family of transcription factors (41). Significant changes in the *ZSCAN12* gene promoter were also seen in URO-MSC52, as well as URO-ASSC cells. The data in Figure 2 further show that the gene promoters that show statistically significant changes that are common to both URO-MSC52 and URO-ASSC are already beginning to show these changes in URO-MSC24.

Figure 2 shows the 57 statistically significant gene promoters sorted by adjusted P-value relative to differences between the three arsenical-exposed cell lines and the parental UROtsa and includes all 17 of

Associated Gene	URO-MSC24	URO-MSC52	URO-ASSC	Adj. p-value
DBC1	0.02	-0.81	-1.02	0.001
TSLRP	-0.06	-1.58	0.10	0.001
ZSCAN12	-0.85	-0.78	-0.87	0.002
PKD2L2	-0.06	-0.33	-1.30	0.004
FAM83A	-0.34	-0.59	-1.06	0.005
KRT7	-0.19	-1.07	-1.33	0.005
C1QTNF6	-0.84	-0.76	-1.09	0.005
LHPP	-0.12	0.11	-0.96	0.005
FGF5	-0.37	-0.72	-1.14	0.006
KCNK10	0.24	0.53	0.78	0.006
FLJ20154	-0.16	-0.98	-0.08	0.007
PLAB	0.13	-0.99	-1.09	0.007
G0S2	-0.67	-0.87	-0.92	0.007
PSMB9	-0.34	-0.29	-0.97	0.010
FLJ11286	-0.11	0.08	-0.67	0.012
FLJ10290	-0.04	-0.86	-0.18	0.012
CPA2	0.03	0.81	0.00	0.013
EYA4	-0.04	-0.47	-0.88	0.015
SPARC	-0.68	-0.71	-0.79	0.015
KLRF1	0.02	-1.29	-0.05	0.015
FLJ11787	-0.47	-0.91	-0.70	0.015
CH25H	-0.58	-0.67	-0.66	0.015
NEFL	0.87	0.66	0.85	0.015
C20orf54	-0.52	-0.78	-0.32	0.020
BRD1	-0.13	0.54	-0.03	0.022
RNF32	0.40	0.43	0.86	0.025
TRPM4	0.20	0.35	0.67	0.025
clg5	0.17	-0.20	0.85	0.025
IFITM2	-0.13	-0.28	-0.86	0.025
HOXp1A10	-0.23	0.58	0.01	0.025
SLC22A7	0.17	0.59	0.07	0.025
TM4SF2	0.32	0.88	0.62	0.025
HOXp1D1	0.09	0.58	-0.20	0.031
HKR3	-0.28	0.44	-0.11	0.031
PAP	0.24	0.64	0.10	0.032
GGA3	0.18	0.64	0.16	0.032
SYT8	-0.10	0.35	-0.38	0.032
CTMP	-0.25	-0.21	-0.68	0.034
AMOT	0.42	0.81	0.63	0.035
SDCCAG28	0.31	-0.58	0.04	0.035
FLJ20333	0.08	-0.41	0.18	0.035
ERBB4	1.08	1.13	0.32	0.036
GSTM4	-0.64	-0.30	-0.19	0.038
ALDH7A1	-0.37	-0.57	0.04	0.043
OSCAR	-0.17	0.42	0.02	0.045
PCDHGB2	-0.26	-0.71	-0.68	0.045
KIAA0277	0.03	0.54	0.33	0.045
FLJ22688	-0.13	-0.60	-0.58	0.045
D123	0.02	0.31	-0.30	0.046
ISG15	-0.37	-0.04	-0.76	0.050
IL11RA	-0.03	-0.49	-0.24	0.050
TEL2	-0.34	-0.36	-0.78	0.050
HHGP	-0.06	-0.40	0.33	0.050
H4FC	-0.26	-0.80	-0.34	0.050
TSSC4	-0.04	0.55	0.20	0.050
V1RL1	0.14	-0.45	0.44	0.050
TGFBI	-0.49	-0.17	-0.78	0.050

Fig. 2. Arsenicals selectively alter histone H3 acetylation. Associated gene names of the significantly affected promoters are shown with promoters validated by real-time PCR shown in bold. Values represent log 2 ratios of each cell line derivative relative to UROtsa. Negative values are representative of hypoacetylated elements, whereas positive values represent hyperacetylated promoters. Promoters are sorted by adjusted P-value relative to differences between all three treated cell lines and UROtsa. All changes represented as significant for that particular cell line when compared with UROtsa (adjusted P-value < 0.05) are shaded. Adjusted P-values were calculated according to the methods described by Benjamini *et al.* (40). For a complete list of promoters exhibiting altered histone acetylation, refer to supplementary Figure 1 (available at *Carcinogenesis* Online).

the gene promoters common to both URO-MSC52 and URO-ASSC. The remaining 43 statistically significant genes (adjusted $P < 0.05$) are provided in supplementary Figure 1 (available at *Carcinogenesis* Online). The values are the log 2 ratios of histone acetylation change for that cell line compared with the UROtsa parent cell line; the shaded values indicate the gene promoters that show statistically significant changes in the given cell lines. Interestingly, the most significant gene promoter identified was for *DBC1*, which stands for 'deleted in bladder cancer', a gene with tumor suppressor function whose activity is frequently lost in bladder as well as other cancers (42,43). Taken together, these data indicate that arsenical-selected malignant transformation may result in non-random, gene-specific changes in histone H3 acetylation and that the genes associated with these promoters could be important participants in arsenical-mediated malignant transformation.

In order to confirm the promoter microarray results obtained, we validated a subset of these genes using the ChIP DNA coupled to real-time PCR. Five promoter regions that showed differential acetylation were selected for confirmatory real-time PCR analysis. Four of these regions (*DBC1*, *FAM83A*, *ZSCAN12* and *C1QTNF6*) showed hypoacetylated histone H3 in the transformed cell lines relative to UROtsa, whereas one showed hyperacetylation (*NEFL*), roughly reflecting the ratio of hypoacetylated to hyperacetylated elements observed in the microarray analysis. PCR primers were designed to amplify a region located within the microarray probe sequence. Equal amounts of input DNA and acetyl-H3-immunoprecipitated DNA were analyzed using promoter-specific primers with the results displayed as the enrichment of each individual sample over its respective input DNA according to the delta Ct method (Figure 3; supplementary Figure 2 is available at *Carcinogenesis* Online). The enrichment of each promoter examined via real-time PCR correlated well with what was observed using the human promoter microarray, showing that the results obtained were reliable. The promoter region of *GAPDH*, an ubiquitously expressed housekeeping gene whose promoter is known to be enriched for acetylated histone H3, was used as a positive control for successful ChIP. All samples showed similar enrichment levels for *GAPDH* (supplementary Figure 3 is available at *Carcinogenesis* Online). Overall, the real-time PCR analysis of selected promoters confirmed the results obtained from the promoter microarray experiments.

Gene expression correlates with the histone H3 acetylation pattern

To determine if the observed changes in histone acetylation are linked to changes in gene expression, we analyzed these genes by quantitative real-time reverse transcription–PCR. The genes *DBC1*, *FAM83A*, *ZSCAN12* and *C1QTNF6* whose promoters showed a decrease in H3 acetylation also showed a corresponding decrease in associated gene expression (Figure 4). In addition, *NEFL*, a gene whose promoter region showed a significant increase in histone acetylation in the arsenical-selected cell lines, also showed an increase in gene expression in these malignantly transformed cells. Taken together, these data suggest that the changes observed in histone acetylation levels are probably closely linked to changes in gene expression in malignantly transformed cell lines, thereby potentially playing a functional role in a cancer-specific fashion.

Since histone acetylation state is an important component that affects gene transcription and is a highly dynamic modification that may be rapidly changed by acute arsenical exposure, we looked to see if 24 h exposure altered the gene expression of a subset of identified genes. To do so, UROtsa cells were exposed to either 50 nM MMA(III) or 1 μM As(III) for 24 h and analyzed for gene expression by real-time reverse transcription–PCR. After exposure, none of the arsenical target genes examined showed changes that approached those seen in the malignantly transformed cell lines, suggesting that these genes are not part of an early arsenical response (supplementary Figure 4 is available at *Carcinogenesis* Online).

Aberrant DNA methylation occurs in the differentially acetylated promoter regions

As histone deacetylation has been mechanistically linked to DNA hypermethylation, we wanted to determine if DNA methylation was increased in the same gene promoter regions that showed changes in histone H3 acetylation. We isolated genomic DNA and performed

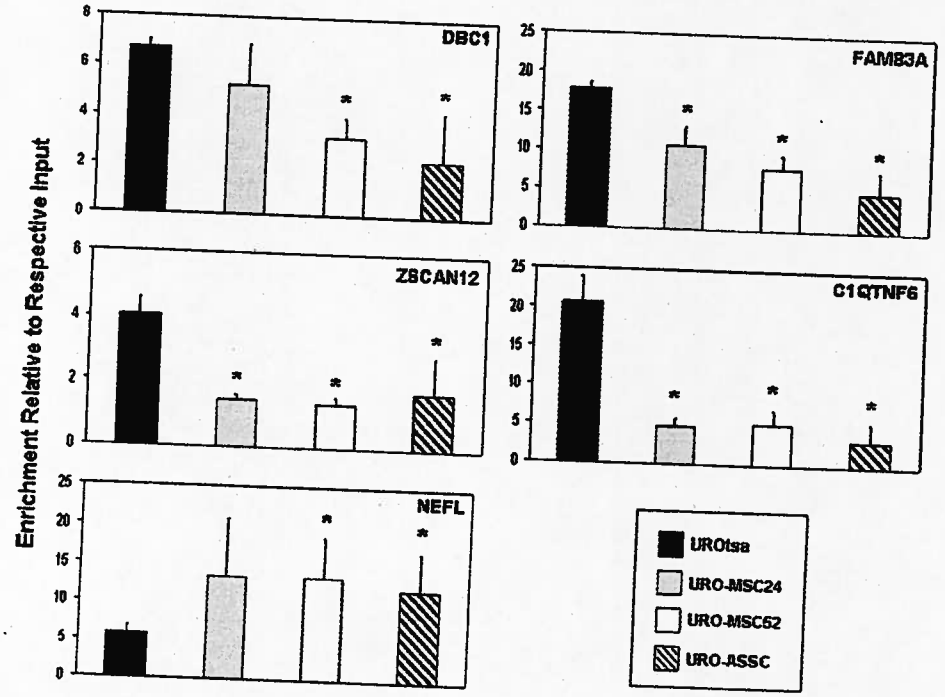

Fig. 3. Real-time PCR confirms the changes in histone H3 acetylation observed using the human promoter microarray. ChIP experiments were coupled to real-time PCR to confirm the histone H3 acetylation levels present with the promoter regions of selected genes in UROtsa, URO-MSC24, URO-MSC52 and URO-ASSC cells. Mean fold enrichment over the respective input DNA for three independent samples is represented by changes along the *y*-axis. Error bars are representative of the standard deviation of three independent experiments. Changes significantly ($P < 0.05$) different than UROtsa are indicated by *.

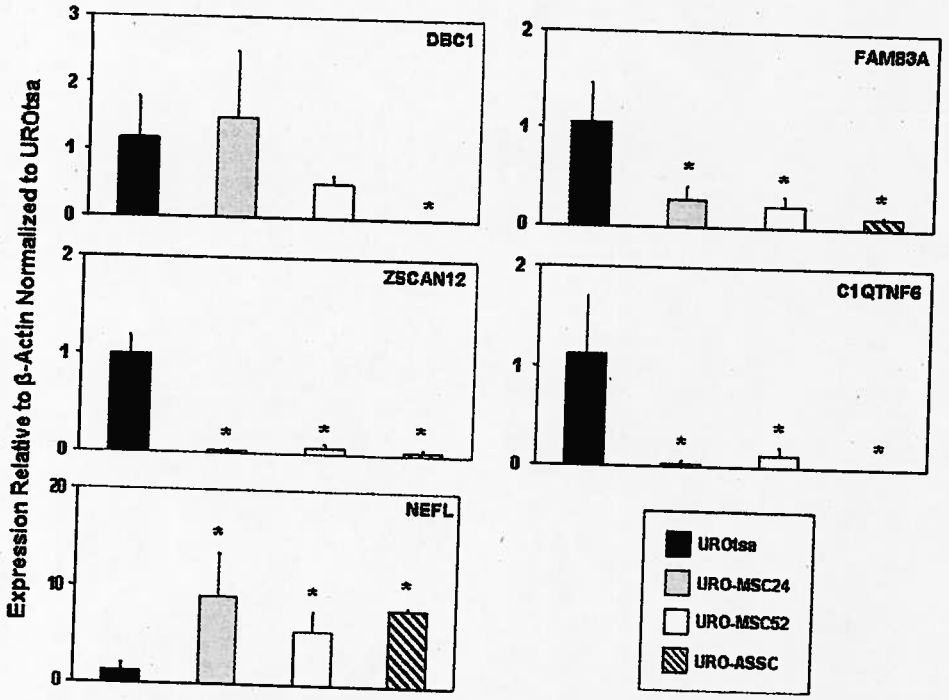

Fig. 4. Gene expression levels correlate with changes in histone acetylation. Expression levels were examined for genes that were verified to have differentially acetylated promoters. Quantitative real-time reverse transcription–PCR was performed on three independent samples for each cell line with the average expression value shown. Expression levels presented on the *y*-axis are relative to β-actin expression and normalized to UROtsa. Error bars are representative of the standard deviation. Changes significantly ($P < 0.05$) different than UROtsa are indicated by *.

immunoprecipitation experiments with an antibody directed toward 5-methylcytosine methylated DNA immunoprecipitation (MeDIP) and coupled this with real-time PCR to assess relative DNA methylation levels. The same primers that were used to examine histone H3 acetylation were used to analyze relative methylation. The DNA methylation level observed in each of these hypoacetylated genes was inversely correlated with the histone acetylation levels at the same locus in the promoter regions of *DBC1*, *FAM83A*, *ZSCAN12* and *C1QTNF6* (Figure 5A). Although not all these changes reached a statistically significant level of $P < 0.05$ when using an unpaired *t*-test, the pattern of increased DNA methylation corresponding with decreased histone H3 acetylation was evident. Conversely, DNA hypomethylation could be associated histone hyperacetylation. DNA methylation levels in the hyperacetylated promoter region of NEFL, however, showed no significant change in DNA methylation between the malignantly transformed cell lines and UROtsa. The promoter region of GAPDH, a gene whose promoter is known to be unmethylated, was used as a negative control. All samples showed similar low levels of enrichment for GAPDH (supplementary Figure 3 is available at *Carcinogenesis* Online). Taken together, these data allow us to conclude that aberrant DNA methylation in selected promoter regions is associated with a decrease in H3 acetylation and decreased gene expression during arsenical-induced malignant transformation.

To verify and extend the DNA methylation data obtained using MeDIP, we performed clonal sodium bisulfite sequencing. We chose to examine a region located within the promoter of *ZSCAN12* (Figure 5B). Results show that the pattern of DNA methylation observed, UROtsa and URO-MSC52 having lower levels of DNA methylation than URO-MSC24 and URO-ASSC, is consistent with MeDIP PCR results. Taken together, these results suggest that the data obtained using MeDIP coupled to real-time PCR are reliable and the promoter region of *ZSCAN12* is hypermethylated in the exposed cell lines.

Histone H3 methylation is altered in a subset of promoter regions examined

To gain a more comprehensive knowledge of the histone modification profile in each of the promoter regions examined, we performed ChIPs to examine the levels of the repressive histone modifications histone H3 lysine-27 trimethylation and histone H3 lysine-9 dimethylation as well as the permissive histone modification histone H3 lysine-4 dimethylation.

While there were statistically significant small changes in histone H3 lysine-27 trimethylation (supplementary Figure 5 is available at *Carcinogenesis* Online), histone H3 lysine-9 dimethylation (supplementary Figure 6 is available at *Carcinogenesis* Online) and histone H3 lysine-4 dimethylation (supplementary Figure 7 is available at *Carcinogenesis* Online) in a subset of promoter regions, the majority of the promoters examined did not exhibit altered levels of histone methylation. It is important to note that while many of these regions do not have significantly altered levels of histone H3 methylation, none of the significant changes measured was discordant with histone H3 acetylation. Although clearly not the case in all the promoter regions measured, examination of the levels of histone H3 histone H3 lysine-27 trimethylation, histone H3 lysine-9 dimethylation and histone H3 lysine-4 dimethylation suggests that, in addition to histone acetylation and DNA methylation, histone H3 methylation may be altered in select promoter regions, providing further evidence of epigenetic remodeling during arsenical-induced malignant transformation.

Pharmacologic reactivation of silenced genes confirms an epigenetic mechanism

Epigenetic modifications are targets of cancer therapy as they are inherently reversible. To confirm that the differential epigenetic profile in the promoter regions of the downregulated genes *DBC1*, *FAM83A*, *ZSCAN12* and *C1QTNF6* was playing a functional role in controlling gene expression, we treated the malignantly transformed URO-ASSC cell line with the DNA methyltransferase inhibitor 5-aza-dCyd, the histone deacetylase inhibitor TSA or a combination treatment with both. Treatment with 10 μM 5-aza-dCyd was performed for 96 h, whereas treatment with 300 nM TSA was performed for 24 h. After the completion of treatment, gene expression levels were measured using real-time reverse transcription–PCR. Each experiment was performed in triplicate to ensure reproducibility.

Pharmacologic reactivation with the epigenetic modifying drugs suggests that the expression of each of the genes examined is

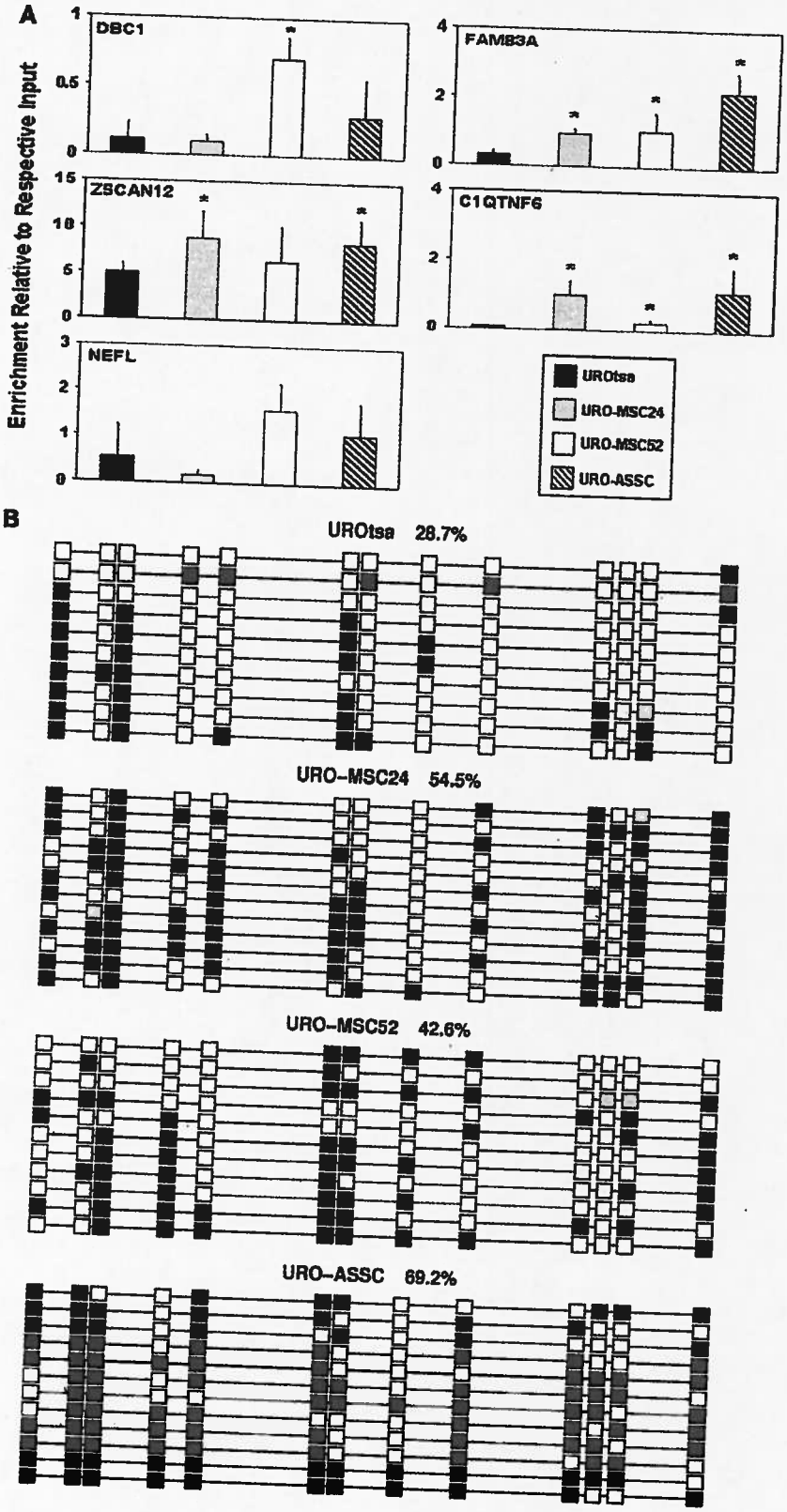

Fig. 5. DNA methylation is altered in selected gene promoter regions. (**A**) Relative DNA methylation levels were measured for the same promoters that were found to be hypoacetylated. MeDIP was performed on three independent samples from each cell line and coupled to real-time PCR with the mean enrichment relative to respective input shown along the *y*-axis. Error bars are representative of the standard deviation. Changes significantly ($P < 0.05$) different than UROtsa are indicated by *. (**B**) Sodium bisulfite sequencing confirms MeDIP results. Sodium bisulfite sequencing was performed to examine the DNA methylation levels and pattern in the promoter region of *ZSCAN12*. Each column represents an individual CpG site, whereas each row is representative of an individual sequenced clone. Spacing is representative of the underlying DNA sequence with the methylation state at each CpG dinucleotide [methylated (filled squares); unmethylated (open squares) and poor sequence (gray squares)] shown. Numbers describe the methylation level for each cell line in the region shown. Clones were sorted for presentation.

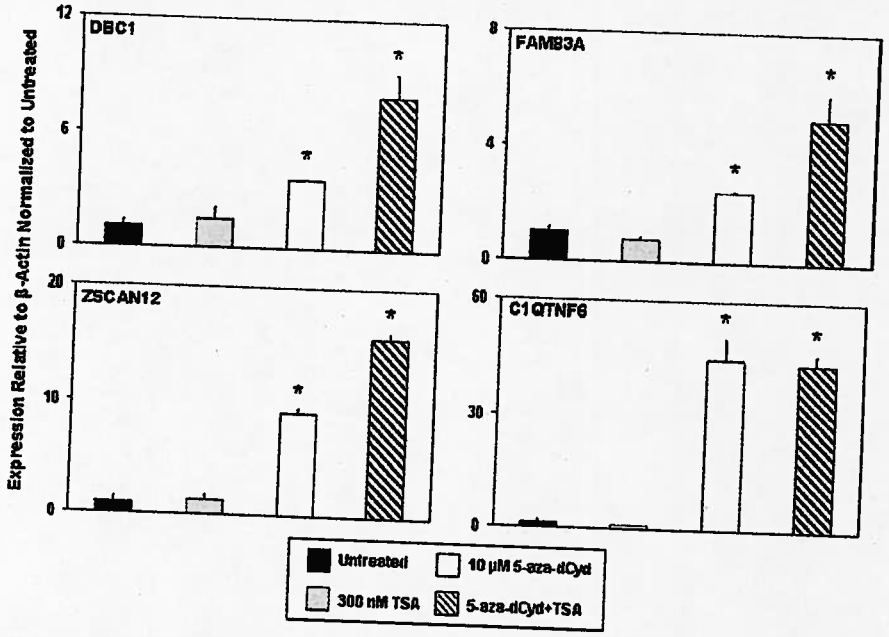

Fig. 6. Pharmacologic reactivation of gene expression using epigenetic inhibitors. URO-ASSC cells were treated with 10 μM 5-aza-dCyd, 300 nM TSA or both. Gene expression levels were measured using real-time reverse transcription–PCR with the values on the *y*-axis of each graph representing expression levels relative to β-actin and normalized to the untreated control. Error bars are representative of the standard deviation of three independent experiments. Changes significantly ($P < 0.001$) different than the untreated control are indicated by *.

mediated by epigenetic mechanisms in URO-ASSC cells (Figure 6). Treatment of these cells with TSA alone did not significantly change the expression of any of the hypoacetylated genes examined. Conversely, treatment with 5-aza-dCyd alone significantly increased the expression of each of these genes ($P < 0.001$). Dual treatment with 5-aza-dCyd and TSA resulted in an additive effect in terms of increasing gene expression. These data suggest that both of these epigenetic marks probably contribute and that epigenetic remodeling has a functional effect in controlling the expression level of associated genes.

Discussion

Arsenic exposure is associated with the etiology of various types of cancer including cancer of the bladder. The mechanisms by which arsenicals result in malignancy include the alteration of signaling pathways and indirect DNA damage, although they are only mildly mutagenic (12–19,24,25). We have demonstrated herein that malignant transformation of human urothelial cells by arsenicals is also associated with changes in histone acetylation and DNA methylation in gene promoter regions. Independent exposures to As(III) and MMA(III) resulted in a non-random perturbation of the epigenomic landscape and allowed us to define genes that may be important in tumorigenesis associated with arsenical exposure. Taken together, these data suggest that epigenetic remodeling is linked to malignant transformation associated with chronic, environmentally relevant exposure to As(III) and MMA(III).

Exposure to heavy metals, in particular nickel, has been shown to alter the histone code (21–23). Our data suggest that this phenomenon also occurs as a result of arsenical exposure. This is supported by a recent study suggesting that exposure to As(III) results in the alteration of multiple methylation marks on histone H3 in a global fashion (44). Microarray experiments showed numerous differentially acetylated promoter regions between the parental and malignantly transformed cell lines. Although many promoter regions were found to be differentially acetylated, it is probably that due to the sensitivity of the microarray and the stringency of our statistical criteria, we are potentially underestimating the number of changes in histone H3 acetylation in these cell lines since we were able to identify statistically significant changes in the URO-MSC24 cell lines utilizing real-time PCR that had not reached statistical significance by microarray.

Aberrant DNA methylation is a hallmark of nearly all types of cancer including bladder tumors and has been functionally linked to histone acetylation (45–48). We performed MeDIP coupled to real-time PCR to determine if relative DNA methylation levels were changed in the promoter regions of genes showing significant decreases in histone acetylation in the exposed cells. Previous studies in rodents demonstrated a loss in genomic DNA methylation in hepatocytes exposed to arsenic (24,25). The data presented here compliment these earlier studies as human tumors generally exhibit genomic hypomethylation while possessing focal regions of DNA hypermethylation, notably in gene promoter regions.

Importantly, the relative amount of DNA methylation increased with decreasing histone acetylation levels in the same promoters, providing additional evidence of the inverse relationship between these two epigenetic marks and further suggesting functional significance of these epigenetic changes. These data suggest the possibility of a mechanism of action of arsenicals whereby they induce yet unknown signaling pathways, resulting in aberrant DNA methylation in critical promoter regions. Methylated DNA can recruit histone deacetylase complexes through the protein MeCP2, resulting in a subsequent loss of histone acetylation in the promoter regions exhibiting increased DNA methylation (47,48). The result of such a mechanism, DNA hypermethylation with a concomitant loss of histone acetylation and corresponding gene expression, is the predominant pattern observed in this study.

This study has uncovered numerous genes that act as epigenetic targets of As(III) and MMA(III) during malignant transformation. These changes occurred in two independent models of arsenical-induced malignant transformation; thus, they may potentially play a role in this conversion, providing targets for future study and therapeutic design. Many of these genes are newly annotated and their cellular functions are not clear. Two genes that are particularly intriguing are *DBC1* and *ZSCAN12*.

Loss of heterozygosity on chromosome 9q is the most common genetic aberration in transitional cell carcinoma, suggesting the presence of a tumor suppressor gene (42). Analysis of this region revealed

the gene to be *DBC1* or deleted in bladder cancer 1. *DBC1* is downregulated by genetic and epigenetic mechanisms in multiple cancers including non-small-cell lung cancer and bladder cancer (42,43). *DBC1* is transcriptionally silenced in some bladder tumor cell lines and this silencing can be reversed after treatment with 5-aza-dCyd, suggesting an epigenetic mechanism of repression (42). The mechanism of action of *DBC1* may involve regulation of cellular proliferation as the reintroduction of this gene induces cell death in bladder cells (49). This possible mechanism is reinforced as URO-ASSC and URO-MSC cell lines exhibit an increased rate of proliferation compared with UROtsa (7,29). The detection of a gene frequently seen to be dysregulated in bladder tumor cells suggests the possibility that this study may not only be important in uncovering genes related to arsenical-induced malignant transformation but also those playing a role in the conversion of a bladder cell to a neoplastic phenotype.

ZSCAN12 is a member of the Kruppel family of zinc fingers proteins. While little is known about this particular gene, zinc fingers typically act as transcription factors, therefore it stands to reason that the downregulation of this gene by arsenicals could affect other genes, hence amplifying its effects. Additionally, the loss of histone acetylation and associated gene expression of *ZSCAN12* is an early event in arsenic selection and may merit further evaluation as a biomarker of precancerous lesions associated with arsenical exposure in the bladder.

In conclusion, this study suggests that the carcinogenic activity of arsenicals may be mediated by the disruption of normal epigenetic control at specific loci. The observed epigenetic changes are correlated with the expression of associated genes, thereby uncovering a collection of genes probably to play an important role in malignant transformation associated with arsenicals. To the best of our knowledge, the data presented herein is the first to show that epigenomic remodeling occurs at specific promoters during arsenical-induced malignant transformation in human cells, providing further understanding of the molecular etiology of arsenical-induced bladder carcinogenesis as well as describing critical genes that may play a role in this process.

Supplementary material

Supplementary Figures 1–7 can be found at http://carcin.oxfordjournals.org/

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Arsenic Toxicology: Translating between Experimental Models and Human Pathology

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Background: Chronic arsenic exposure is a worldwide health problem. How arsenic exposure promotes a variety of diseases is poorly understood, and specific relationships between experimental and human exposures are not established. We propose phenotypic anchoring as a means to unify experimental observations and disease outcomes.

Objectives: We examined the use of phenotypic anchors to translate experimental data to human pathology and investigated research needs for which phenotypic anchors need to be developed.

Methods: During a workshop, we discussed experimental systems investigating arsenic dose/exposure and phenotypic expression relationships and human disease responses to chronic arsenic exposure and identified knowledge gaps. In a literature review, we identified areas where data exist to support phenotypic anchoring of experimental results to pathologies from specific human exposures.

Discussion: Disease outcome is likely dependent on cell-type–specific responses and interaction with individual genetics, other toxicants, and infectious agents. Potential phenotypic anchors include target tissue dosimetry, gene expression and epigenetic profiles, and tissue biomarkers.

Conclusions: Translation to human populations requires more extensive profiling of human samples along with high-quality dosimetry. Anchoring results by gene expression and epigenetic profiling has great promise for data unification. Genetic predisposition of individuals affects disease outcome. Interactions with infectious agents, particularly viruses, may explain some species-specific differences between human pathologies and experimental animal pathologies. Invertebrate systems amenable to genetic manipulation offer potential for elaborating impacts of specific biochemical pathways. Anchoring experimental results to specific human exposures will accelerate understanding of mechanisms of arsenic-induced human disease.

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Arsenic exposure via drinking water affects > 140 million people worldwide and causes cancer and bronchopulmonary, cardiovascular, and metabolic diseases and neuropathies. Various experimental models have been developed to understand how arsenic exposure causes these diverse disease outcomes. Translation of laboratory arsenic toxicology studies to human health is important but is complicated by inexact dose conversion between *in vitro*, murine, and human exposures and species-specific metabolic differences. Here, we discuss issues in dose conversion and potential means to translate findings in selected experimental model systems to an understanding of human arsenic toxicology. Phenotypic anchoring of results from model systems by tissue dosimetry, gene expression and epigenetic mark profiling, and tissue biomarker identification should promote development of a coherent picture of mechanisms of arsenic-induced human disease. We discuss research needs critical to progress in translation of experimental findings. We also highlight a human-specific disease end point and discuss advantages of invertebrate systems to address specific questions in a simpler background with fewer confounding factors.

Dose and Exposure Conversion

Data collected in human studies often include exposures but not doses. Urine and toenail arsenic are often used as indicators of body burden but are subject to wide individual variation with similar exposures. Dose conversion between human and murine exposures is a complicated issue. Calculating dose requires careful determination of amounts consumed and is rarely reported. Often, consumption estimates are based on data from published studies. However, water consumption can vary greatly in mice and is markedly different in different strains (Bachmanov et al. 2002). Likewise, human exposure data include an estimate of arsenic-contaminated water and/or food consumption. However, body weights are not systematically collected and differ greatly with study population. Hence, calculation of human dose with individual precision has not been done. Even with reliable dose estimates, dose conversion between the mouse and human is complicated. An estimate based on body surface area may be reliable for many substances (Reagan-Shaw et al. 2008), but arsenic metabolism is strikingly different in rodents and humans (Vahter 1999). For these reasons, anchoring results by induced phenotype may be a more useful approach. A simple anchor might be target tissue arsenic levels. Murine tissue dosimetry can be performed readily, although most data currently available are from mice with high arsenic exposures (Devesa et al. 2006; Gentry et al. 2005). Some human data on tissue, blood, and urine arsenic levels have been correlated with exposures in specific populations. Thus, this approach is limited in that data available are on a population level, but there are no systematic compilations of these correlations on an individual level. Hence, no direct connection between a specific human exposure and a biological arsenic level is available, and research including these measures is needed. Other approaches to determine exposure equivalence by induced phenotype include anchoring by changes in gene expression, epigenetic marks, or tissue remodeling biomarker profiles. These approaches are certainly possible within laboratory models and could readily serve to unify results from experimental systems. However, only very limited data sets are available for human exposures. Thus, there is a great need for research collecting these data from humans

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who exhibit arsenic-induced disease. These data are critical to translation of experimental results to specific human exposures.

Transplacental exposures. *In utero* exposures to environmental toxicants can have a profound effect on development of chronic adult diseases. Endocrine disruptors are paradigms of developmental toxicants and are linked to diseases as diverse as prostate cancer (Ho et al. 2006) and obesity (Grun and Blumberg 2009). Consequences of *in utero* arsenic exposure in humans are difficult to determine in most cases because exposure is not limited to the *in utero* period but continues into postnatal life. However, a unique situation with a defined period (1958–1971) of arsenic exposure occurred in Antofagasta, Chile (Borgono et al. 1977). This unfortunate incident provides a cohort with a defined period of exposure. Increased incidence of a variety of disease conditions associated with the arsenic exposure was reported shortly after the switch back to low-arsenic water (Borgono et al. 1977). These conditions included increased incidence of bronchopulmonary and cardiovascular diseases, both now clearly linked to chronic arsenic exposure (Argos et al. 2010). Long-term follow-up studies of this cohort revealed high mortality from lung cancer and bronchiectasis in the population exposed *in utero* and during early childhood decades after high exposure ended (Smith et al. 2006). Additionally, the incidence of myocardial infarction in infants whose mothers were exposed during this period (Rosenberg 1974) indicates that *in utero* arsenic exposure could induce cardiovascular disease.

In contrast to the striking results from the Antofagasta population, infant mortality—but not spontaneous abortion—showed dose correlation in a Bangladeshi population (Rahman et al. 2010). This difference may be due to a difference in exposure levels. The water arsenic level in Antofagasta during the high exposure period was approximately 800 μg/L and uniform in the population because there was a single source of water, whereas the Bangladeshi population experienced variable exposures due to multiple sources: 268–2,019 μg/L (median, 390 μg/L) for infant mortality and 249–1,253 μg/L (median, 382 μg/L) for spontaneous abortion study populations. Taken as a whole, *in utero* exposure to high levels of arsenic in drinking water appears to be necessary for obvious adverse effects early in postnatal life. It is likely that lower exposures have a more subtle effect, perhaps contributing to chronic adult diseases.

High arsenic exposure *in utero* affects gene expression in leukocytes from human cord blood (Fry et al. 2007). Gene ontology analysis of altered mRNA expression in arsenic-exposed samples revealed that immune, inflammatory, and stress response categories were affected. Network analyses identified JUNB, interleukin (IL) 8, IL1β, and hypoxia-inducible factor-1α, which are involved in cell cycle regulation, stress response, inflammation, and response to hypoxia, respectively. In addition, nuclear factor-κB was integrated into the subnetworks and also found to be activated in the cord blood of arsenic-exposed infants.

Animal studies indicate that *in utero* arsenic exposures induce both cancer and atherosclerosis. *In utero* arsenic exposure (42.5 or 85 ppm) induced tumors in C3H mice (Waalkes et al. 2003) and established the first reproducible laboratory animal model of carcinogenesis by inorganic arsenic (iAs) alone. Recent work from this group shows that whole-life exposure at lower levels (6–24 ppm) results in higher tumor incidence (Tokar et al. 2011). Combined with *in vitro* studies showing enhanced proliferation of stem cells, these results led to the hypothesis that cancer induced by *in utero* arsenic exposure is a consequence of arsenic-induced increase in the stem cell population of target tissues (Tokar et al. 2010).

Both *in utero* (Srivastava et al. 2007) and postweaning (Srivastava et al. 2009) exposures to arsenic in drinking water accelerate and exacerbate atherogenesis in the apolipoprotein E-knockout ($ApoE^{-/-}$) mouse model for atherogenesis. These studies showed that atherogenesis was induced by arsenic exposure alone, without the high-fat diet normally used to induce atherosclerosis in this model. The *in utero* arsenic exposure (49 ppm) used in the $ApoE^{-/-}$ experiments produces arsenic levels in livers of the pregnant dams (States JC, unpublished data) similar to those observed in livers of people exposed to high levels of arsenic (200–600 ppb) in drinking water in West Bengal (Guha Mazumder 2001). Data from gene expression analyses show induction of immune, inflammatory, and stress response pathways in livers of 10-week-old $ApoE^{-/-}$ mice exposed to arsenic *in utero* (States JC, unpublished data). These pathways were among the top pathways activated in human cord blood lymphocytes discussed above. Hence, the data suggest that these responses are induced in multiple tissues and may be a common basis from which disease processes emerge. Thus, correlation exists in phenotypic anchors (tissue arsenic levels, altered gene expression) between the higher arsenic exposures in Chile and South Asia and these mouse exposures.

Arsenic-induced tissue remodeling. Adverse health effects of chronic arsenic ingestion on the lung include chronic obstructive pulmonary disease, chronic bronchitis, and bronchiectasis. In separate studies in West Bengal and Bangladesh, chronic arsenic exposure reduced lung function (De et al. 2004; Parvez et al. 2008; von Ehrenstein et al. 2005) and increased respiratory disease symptoms (i.e., cough, chest sounds, shortness of breath) and chronic bronchitis (Mazumder et al. 2005; Milton and Rahman 2002). More than 63% of subjects with mean arsenic exposure of 216 ± 211 ppb (compared with 11 ± 20 ppb in controls) displayed increased respiratory complications (Islam et al. 2007). Clearly, high-level arsenic exposure (200–1,000 ppb) causes adverse respiratory effects. However, effects of lower exposures are not known.

Airway remodeling is a hallmark of many respiratory diseases, including emphysema, asthma, idiopathic pulmonary fibrosis, and bronchiectasis (Jeffery 2001; Niimi et al. 2005; Reynolds et al. 2005). Persistent structural changes in tissue develop through a process of injury and dysregulated repair, leading to chronic inflammation and altered extracellular matrix deposition in the airway wall, eventually obstructing airflow. Chronic lung disease phenotypes in populations with high arsenic exposure suggest that extracellular matrix, aberrant cell motility, and wound repair are arsenic targets. Data support this hypothesis because changes in expression and organization of extracellular matrix genes and in expression of mediators and enzymes that control matrix remodeling have been observed consistently in a wide range of model systems.

Expression of a large number of extracellular matrix genes was altered in adult male C57Bl/6 mice exposed to either 10 or 50 ppb arsenic in their drinking water for up to 8 weeks (Lantz and Hays 2006). These alterations included suppression of several collagen, elastin, and fibronectin isoforms. In addition, mRNA for matrix metalloproteinase-9 (MMP-9), a matrix degradation enzyme, was induced. Disorganization and expansion of elastin and collagen after 8-week 50 ppb arsenic exposure were observed around pulmonary airways and blood vessels. Arsenic-induced changes in adult animals also occurred in the extravascular matrix of small cardiac arteries (Hays et al. 2008).

Matrix is also critical for cell migration, wound repair, and remodeling after injury. Pathway analysis using gene and protein expression data from multiple model systems suggests that wound repair and cell motility are two of the more probable processes affected by arsenic exposure (Lantz and Hays 2006; Lantz et al. 2007, 2009; Petrick et al. 2009). Arsenic increased time to close a scratch wound in confluent human airway epithelial cells. This increased closure time (reduced wound repair) was associated with increased expression and activity of MMP-9. Arsenic, even in the absence of the wounding, induced significant production of MMP-9, and inhibition of MMP-9 partially restored repair. Inhibition of repair also occurs in an animal model. Animals exposed to arsenic had less capacity to repair naphthalene-induced airway injury (Lantz RC, unpublished data).

During fetal and early postnatal lung development, extracellular matrix gene expression is necessary for proper development of lung and blood vessels. In the highly exposed Antofagasta population, *in utero* and early postnatal exposure (~ 800 ppb) increased risk of chronic obstructive pulmonary disease and bronchiectasis (Smith et al. 2006). After *in utero* and early postnatal exposure in mice (≤ 100 ppb), lung collagen type 1 α2 (*Col1a2*), *Col3a1*, and elastin mRNA expression increased and exhibited both developmental time and exposure dependence (Lantz et al. 2009). Changes in matrix protein expression may result from arsenic interaction with normal developmental processes. However, whole-lung collagen and elastin levels were not significantly altered. Increased mRNA expression could be a compensatory response. For example, arsenic-induced increases in MMP-9 during early postnatal periods, as seen in a mouse model, would degrade matrix, requiring increased mRNA expression to maintain appropriate protein levels.

Although whole-lung levels of matrix proteins were unchanged, regional decreases in total collagen in adventitia around airways were seen in 28-day-old mice exposed to arsenic during development (Lantz et al. 2009). Localized decreases in collagen were associated with increased levels of smooth muscle around airways and alterations in pulmonary function. Understanding mechanisms for localized arsenic effects requires research.

Of critical importance is whether changes seen in model systems replicate events in human populations. Levels of MMP-9 and its inhibitor, TIMP-1 (tissue inhibitor of metalloproteinase-1), determined in populations with low exposures to arsenic (< 20 ppb) through drinking water showed that the MMP-9:TIMP-1 ratio in induced sputum was positively associated with total urinary arsenic (Josyula et al. 2006). Although the underlying mechanism is different from that in model systems (increased ratios were due predominantly to TIMP-1 decreases in humans), the underlying effect—increased degradation of matrix—is the same.

Thus, ingested arsenic alters matrix and matrix-associated proteins in a number of model systems and in humans. Evaluation of arsenic-induced phenotypic alterations, including lung function, lower respiratory infections (predicted from model systems), and changes in mediators affecting matrix deposition, is needed, especially in children. Changes in matrix deposition may be a source of useful tissue biomarkers for phenotypic anchoring.

Arsenic-induced vascular disease in adult animal models. Arsenic exposure is strongly associated with increased cardiovascular disease risk (States et al. 2009). High exposures cause occlusive arteriosclerosis, such as blackfoot disease seen in Taiwan (Tseng 2008) and coronary occlusion in infants in Chile (Rosenberg 1974). Many studies have found increased cardiovascular disease risk with more modest exposures (10–100 ppb). In the United States, mortalities from vascular diseases were increased in counties where arsenic levels were > 20 ppb relative to those with < 10 ppb (Engel and Smith 1994). Diseases associated with these lower exposures include coronary artery and ischemic heart disease, carotid atherosclerosis, microcirculatory defects, and prolonged QT intervals (Medrano et al. 2010; Tseng 2008; Wang et al. 2009). Arsenic may increase associated vascular disease risk factors, such as systolic hypertension (Chen et al. 2009; Tseng 2008) and diabetes (Navas-Acien et al. 2009). Increased systolic hypertension (Chen et al. 2009) is consistent with direct stimulatory effects of arsenic on vascular smooth muscle (Soucy et al. 2004) and decreased vasorelaxation (Srivastava et al. 2007, 2009). Nutritional (Chen et al. 2009), metabolic (Mazumder et al. 2005; Navas-Acien et al. 2005), and genetic susceptibilities (States et al. 2009) to cardiovascular pathologies caused by arsenic implicate enhanced oxidant signaling as a primary mode of action. This appears as endothelial cell dysfunction and metabolic dysregulation from loss of nitric oxide or gain of oxidant signaling (States et al. 2009).

Mice may be as sensitive as or more sensitive than humans to vascular pathologies caused by low to moderate arsenic exposures. Angiogenesis, tumor angiogenesis, and liver sinusoidal vessel remodeling occur in C57BL/6 mice exposed for 2–5 weeks to 1–10 ppb arsenic (Soucy et al. 2003, 2005; Straub et al. 2008). Mouse models reproduce the atherogenic effects of arsenic after *in utero* (Srivastava et al. 2007) or adult arsenic exposures (Bunderson et al. 2004; Srivastava et al. 2009). In the mouse heart, arsenic caused perivascular fibrosis (Hays et al. 2008) and increased expression of matrix remodeling proteins (e.g., Serpine1 and MMP-9) (Soucy et al. 2005). At higher exposures, progressive loss of myocardial microvessels (Soucy et al. 2005) and cardiomyopathy (Li et al. 2002) occurred. In the developing chicken heart, arsenic affects epithelial to mesenchymal transitions necessary for valves to develop (Lencinas et al. 2010). Arsenic causes liver steatosis, fibrosis, and portal hypertension in humans (Mazumder 2005) that may predispose individuals to risk of systemic atherosclerosis and metabolic disease (Targher et al. 2010). Arsenic causes mouse liver sinusoidal endothelial cell (LSEC) capillarization and periportal vessel hyperplasia (Straub et al. 2007, 2008) that resemble similar pathology seen in infants who died from *in utero* or perinatal arsenic exposures (Rosenberg 1974). As in humans, studies in rabbit models (Pi et al. 2003) and mouse models (Bunderson et al. 2004; Straub et al. 2008) implicated nitric oxide loss and increased oxidant signaling in promoting endothelial cell dysfunction and pathogenic phenotypic change. Thus, animal models recapitulate pathogenic end points that are relevant to arsenic-induced human cardiovascular diseases, and these end points provide phenotypic anchors for systematic investigation of pathogenic mechanisms.

Phenotypic anchors of vessel remodeling and vessel cell-to-matrix interactions involved in remodeling reveal critical signaling pathways underlying the etiology of arsenic-related vascular diseases. Matrix interactions are critical for maintaining vessel integrity, wall cell phenotype, and functional signaling. In a model of epithelial to mesenchymal transition in heart valve development, transcriptomic analysis revealed 382 genes that were responsive to 25 ppb arsenic (Lencinas et al. 2010). Pathway analysis identified clusters of responsive genes involved in cytoskeletal regulation, matrix deposition, and cell adhesion, as well as in stabilizing an endothelial cell phenotype (Lencinas et al. 2010). The cluster of cytoskeletal-regulating genes included GTPases (*Rac1* and similar members of the RhoA GTPase family) known to be activated by arsenic in vascular dysfunction (Qian et al. 2005; Smith et al. 2001; Straub et al. 2007, 2008) and inflammation (Lemarie et al. 2008). *In vivo*, arsenic exposure results in membrane localization of Rac1 in capillarized LSEC (Straub et al. 2007, 2008). In *ex vivo* studies, arsenic-induced LSEC capillarization was prevented by inhibiting Rac1 activity (Straub et al. 2007, 2008). Rac1 also is highly expressed in skin tumors induced by arsenic plus phorbol ester in Tg.AC mice (Waalkes et al. 2008).

The Rac1 signaling program mediates arsenic-induced generation of reactive oxygen species that are second messengers for its pathogenic effects. Rac1 is an essential subunit of Nox2-type NAPDH oxidase, and this oxidase is required for arsenic-stimulated large-vessel endothelial and LSEC oxidant production (Smith et al. 2001; Straub et al. 2008). Arsenic does not capillarize LSEC in mice lacking this oxidase (Straub et al. 2008). This finding was the first *in vivo* demonstration of a role for NADPH oxidase in arsenic action and the first demonstration that the activation of the oxidase promotes LSEC capillarization. In a recent study Ghatak et al. (2010) confirmed that NADPH oxidase activity is central to arsenic-induced liver fibrosis. Further, chronic activation of Rac1 and Nox2-type NADPH oxidases are longitudinal risk factors for vascular disease and hypertension (Lee and Griendling 2008). Gain-of-function polymorphisms in oxidase subunit genes are associated with cardiovascular disease in general (San et al. 2008) and with arsenic-induced disease (States et al. 2009).

There is a significant knowledge gap in understanding how phenotypic change in individual cell types relates to pathogenic vascular remodeling and function. Arsenic-induced LSEC capillarization limits the removal of lipoproteins, lipids, and waste proteins from the circulation and alters normal liver lipid metabolism (Straub et al. 2008). In addition, zonal distribution of hepatocyte lipid deposition changes from being exclusively within hepatocytes surrounding the central veins (zone 3) to spreading into hepatocytes surrounding the portal veins (zone 1). These effects may translate to both liver and systemic vascular diseases (Targher et al. 2010). Arsenic-induced change in liver cell phenotype and underlying cell matrix appears to alter basic liver structure, function, and metabolism. However, full investigation of the LSEC responses is hindered by the overwhelming mass of hepatocytes masking these responses. Preliminary evaluation of total mouse liver mRNA, microRNA, and proteome responses to lower level arsenic exposures revealed modest changes (Straub et al. 2009). This modest effect is expected because there is little observable arsenic-induced change in the hepatocytes. Examination of primary LSEC exposed to arsenic *ex vivo*, however, demonstrated much greater responses that supported the pathogenic *in vivo* effects (e.g., decreased expression of the scavenger receptor stabilin-2). The challenges are to determine whether LSEC-specific or vascular-cell–specific changes can provide markers for arsenic-induced pathogenesis and whether preventing arsenic effects in LSECs or vascular cells prevents systemic pathogenesis. Similarly, there is a need to understand how arsenic-induced change in microvascular phenotype affects organ function, such as in the liver, or systemic metabolic changes that promote cardiovascular and metabolic diseases.

Epigenetic effects of arsenic exposure. The disruption of normal epigenetic control can participate in the etiology of complex human diseases, including psychiatric disorders, cardiovascular disease, diabetes, and cancer. In cancer, pathologic disruption of the normal epigenetic state of a cell can be caused by diverse mediators and mechanisms, including environmental agents, stresses, and cues. Accumulating evidence indicates that arsenic is an environmental toxicant that can mediate epigenetic changes (Reichard et al. 2007; Ren et al. 2011b). Thus, epigenetic control mechanisms are a nexus of gene–environment interactions that link cellular responses to arsenic exposure. DNA and histone modification enzymes and the cellular pathways that input signals to them represent potential targets for disruption leading to an altered epigenetic state and phenotype.

Recent work links arsenic exposure to epigenetic state disruption and progression of the diseased state. During carcinogenesis, arsenic exposure induces global DNA hypomethylation with hypomethylation frequently found in repetitive elements, although DNA demethylation of some gene regulatory regions also occurs (Chen et al. 2004; Jensen et al. 2009a; Reichard et al. 2007). The functional consequences of this DNA hypomethylation remain unclear but may involve inappropriate gene activation or altered chromatin structures. Because arsenicals inhibit activity of DNA methyltransferases DNMT1 and DNMT3a (Reichard et al. 2007), this effect may contribute to overall decreased levels of DNA methylation. However, it may be only one of multiple factors contributing to arsenical-induced epigenetic change, because arsenicals also mediate a coincident DNA hypermethylation of CpG island gene promoters, as well as changes in histone post-translational modifications.

Aberrant DNA hypermethylation of CpG island gene promoters is functionally linked to inappropriate transcriptional silencing, and disease progression. This epigenetic lesion has been found in multiple human cell models of arsenical-induced malignant transformation (Cui et al. 2006; Jensen et al. 2009a). In one example, both arsenite and monomethylarsonous acid (MMA^{III}) induced malignant transformation of an immortalized urothelial cell line model of human bladder cancer (UROtsa) (Bredfeldt et al. 2006; Sens et al. 2004). In this model, arsenite and MMA^{III} each induced hundreds of DNA methylation changes across the genome, with a striking overlap in genes targeted by these similar but chemically distinct arsenicals. These results suggest that different forms of arsenic may act similarly in their ability to perturb the epigenetic landscape. For example, in the UROtsa model, both MMA^{III} and arsenite induced DNA hypermethylation-associated gene silencing of *DBC1* (deleted in bladder cancer 1) and *G0S2* (G0/G1 switch regulatory protein 2) (Jensen et al. 2008, 2009a). Interestingly, both of these genes display tumor suppressor function and become aberrantly methylated and transcriptionally silenced in clinical bladder cancer (Chang et al. 2010; Habuchi et al. 1998; Hoque et al. 2006; Izumi et al. 2005; Kusakabe et al. 2010; Welch et al. 2009), suggesting that *in vitro* models of arsenical-induced malignant transformation may accurately reflect epigenetic events that occur in clinical disease. The human relevance of these *in vitro* studies is further suggested by recent human population-based studies that found a connection between arsenic exposure and epigenetic dysfunction in bladder cancer (Marsit et al. 2006).

Many of the arsenic-mediated epigenetic gene-silencing events linked to gene promoter DNA hypermethylation were also accompanied by changes in the histone code in these same regions, specifically hypoacetylation of histones H3 and H4 (e.g., Jensen et al. 2009a). The temporal order of and mechanisms involved in this multifaceted epigenetic reprogramming are not clear. The epigenetic state change may result from a new epigenetic program being enacted by arsenical-driven alterations in cell signaling inputs. Alternatively, arsenicals may act on multiple epigenetic modifier enzymes to short-circuit the epigenetic program. Indeed, changes in both histone phosphorylation and histone methylation that appear independent of DNA methylation changes occur after arsenical exposure (Jensen et al. 2009b; Zhou et al. 2008). Taken together, these results indicate that arsenicals likely disrupt multiple epigenetic pathways.

Epidemiological studies of Chilean populations show an arsenic-related increase in lung and bladder cancer mortality, as well as a long latency between the time of major arsenic exposure and increased disease rates (e.g., Marshall et al. 2007). The long latency suggests that arsenicals may damage the epigenomic integrity of progenitor or stem cell populations and that the expanded populations arising from these progenitors retain the epigenetic changes. This type of epigenetic initiation event is consistent with the first step in the recently proposed epigenetic progenitor theory of carcinogenesis (Feinberg et al. 2006). Specifically, we predict that arsenicals induce changes in the epigenetic terrain of progenitor cells that are faithfully inherited from cell generations, even after removal of the initiating toxicant. Thus, arsenicals may act as epimutagens—agents capable of altering the epigenome of cell populations, resulting in changes in gene expression and phenotypic shifts. This long-term epigenetic damage may remain silent until other critical events occur (e.g., loss of *p53*, immortalization), at which time the arsenical-induced epigenetic changes may be phenotypically "unmasked" and help drive evolution of the malignant phenotype (e.g., suppression of tumor suppressor genes). The precise mechanisms responsible for arsenic's disruption of a cell's epigenetic state are being elucidated and will be critical for a full understanding of arsenical action. Research profiling epigenetic changes in human tissues is needed to validate the epigenetic changes observed *in vitro*.

Cutaneous effects of arsenic and human papilloma viruses. In humans, skin is the most sensitive target organ for chronic arsenic exposure (Yoshida et al. 2004). Even at low-level exposures, arsenic increases risks for pigmentation changes (melanosis), hyperkeratosis, Bowen's disease, and nonmelanoma skin cancer (NMSC) (Agency for Toxic Substances and Disease Registry 2007; Chen et al. 2009). Although chronic arsenic exposure is causally

linked with skin disease, cutaneous arsenicosis is solely a human disorder for reasons that remain unknown (Rossman et al. 2002). However, human-specific hyperkeratosis may be linked to enhanced viral infection and immune suppression observed in laboratory studies.

One possible explanation for the human specificity of the effect of arsenic on skin is an interaction with a viral skin pathogen. Arsenic exposures inhibit immune function, at least in part by inhibiting immune surveillance of dendritic cells and CD4 cell activation (Lantz et al. 1994; Liao et al. 2009). By compromising immune function, arsenic impairs the immune response to viruses. This effect has been demonstrated for influenza A, for which arsenic exposure elevates viral titers and increases morbidity (Kozul et al. 2009; Yu et al. 2006). Similarly, it has been known for more than a century that arsenic exposure can reactivate latent herpes infections (Au and Kwong 2005; Lanska 2004). Likewise, human papillomavirus (HPV), a human-specific pathogen, shares several clinical features with arsenicosis and may contribute to arsenical skin disease. Cutaneous HPV establishes infection by evading detection by skin dendritic cells (Langerhans cells). Therefore, it is reasonable that immune inhibition by arsenic could unmask preexisting infections or impair the immunologic response to new exposures (Frazer et al. 1999).

Most individuals are exposed to dermal HPV during their lifetimes (Pfister 2003). In fact, many individuals have antibodies against HPV, thereby demonstrating prior exposure (Masini et al. 2003). Such exposures may be of little consequence for individuals with normal immune function; however, individuals with impaired immune function are at significantly increased risk of HPV infection and NMSC. Patients with epidermodysplasia verruciformis have an immune defect that prevents recognition of HPV, resulting in severe skin infection and a 90% increase in NMSC risk (Pfister 2003). Likewise, immunosuppressive therapy increases the risk of skin warts and premalignant actinic keratoses 2-fold and risk of squamous cell carcinoma 150-fold (Shamanin et al. 1996; Stockfleth et al. 2004). Thus, arsenic-induced immune suppression may increase HPV infectivity.

Only a handful of studies have investigated the occurrence of HPV infection in dermal arsenicosis. Ninety NMSC patients recruited from an arsenic-endemic region of Mexico were evaluated for the serological presence of HPV-16–reactive antibodies (Rosales-Castillo et al. 2004). The odds ratios for NMSC in patients with a positive history for high arsenic exposure or the presence of antibodies against HPV were 4.53 and 9.04, respectively. This risk increased to 16.5 when both high-level arsenic exposure and HPV were present (Rosales-Castillo et al. 2004). Although it has not been systematically investigated, several case studies have directly detected HPV infection in arsenical skin lesions. HPV types 16 and 41 have been detected in squamous cell carcinomas taken from arsenic-exposed patients (Grimmel et al. 1988; Neumann et al. 1987), and HPV-23 was identified in multiple hyperkeratotic papules from a single patient (Gerdsen et al. 2000). Somewhat in contrast with these findings, Ratnam et al. (1992) detected HPV in only 2 of 33 arsenical keratoses isolated from four patients. The differences among these findings are not surprising given the small study size, the > 100 types of HPV, and the technical challenge associated with broadly detecting cutaneous HPV types (Dang et al. 2006; Vasiljevic et al. 2007).

In addition to arsenic's effect on immune function, arsenic may promote integration of HPV DNA into the genome of keratinocytes, the process underlying HPV-mediated neoplasia (Jones and Wells 2006). Damage to episomal HPV DNA, such as that caused by oxidative stress, is a critical step triggering genomic integration of the virus and expression of genes that promote keratinocyte proliferation and inhibit differentiation (Jones and Wells 2006). By promoting integration, arsenic may enhance the tumorigenicity of HPV (Germolec et al. 1997; Milner 1969; Rossman 1998). Together, HPV and low-concentration arsenic may target epidermal stem cells to promote keratinocyte proliferation and inhibit normal differentiation (Egawa 2003; Liu et al. 2010). Although the effect of arsenic on HPV-infected cells is unknown, preliminary data suggest that arsenic increases cell division and delays differentiation of HPV-infected keratinocytes in organotypic skin cultures, leading to delayed differentiation, increased suprabasal cell division, and suprabasal skin thickening (Reichard JF, unpublished data). Clearly, more research on arsenic enhancement of viral infections in both animals and humans is needed.

Metabolism, genetics, and model systems. Human dose dependence for any arsenic-linked phenotypic outcome depends on multiple critical factors, such as intracellular chemical transformation, tissue distribution, reactivity, and efflux (Thomas 2007). Each can be affected by individual genetic variability, so departures from the "norm" in dose responsiveness and outcome often occur. Use of genetically manipulable models can undoubtedly enhance our understanding of these processes and their importance to toxicity mechanisms.

The methylated derivatives monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) were believed to be detoxified metabolites (Vahter 1999). However, detection of the methylated As^{III} (+3 oxidation state) species in urine (Le et al. 2000) altered perceptions because MMA^{III} is significantly more toxic than either iAs or the other metabolites (Petrick et al. 2000; Styblo et al. 2000). Some 10–20% of urinary metabolite in humans is MMA [much higher than for most mammals (Vahter 2002)]; the expectation that a portion of this is MMA^{III} might account for higher human susceptibility to pathologic outcomes compared with rodents. Studies of arsenic-exposed populations link urinary MMA levels and individual susceptibility to a range of arsenic-related pathologies (Smith and Steinmaus 2009). The genetic contribution to this association is important, with data suggesting that several pathways might contribute to differential MMA levels (e.g., uptake, one-carbon metabolism, speciation, efflux). The *S*-adenosylmethionine–dependent enzyme arsenic (+3 oxidation state) methyltransferase (AS3MT) is capable of transforming iAs to produce MMA and DMA species of both +3 and +5 oxidation states (Li et al. 2005; Thomas et al. 2004). Certain intronic and extragenic *AS3MT* polymorphisms (along with more extended local haplotypes) are associated with higher DMA:MMA ratios (Gomez-Rubio et al. 2010; Schlawicke et al. 2009), whereas the exon 9 polymorphism M287T is associated with higher urinary levels of MMA (Hernandez and Marcos 2008). Recently, this M287T allele was associated with both elevated damage to DNA (Sampayo-Reyes et al. 2010) and enhanced premalignant skin lesions (Valenzuela et al. 2009), suggesting a mechanistic connection to higher MMA levels. More detailed study of the catalytic properties of AS3MT alleles and their response to input from other intersecting pathways (e.g., one-carbon metabolism, redox environment, feedback inhibition) is required.

In the larger context, more insightful studies into the mechanisms and consequences of arsenic uptake, speciation, distribution, retention, and efflux *in vivo* are necessary. Reports on metabolite-specific transport into and out of cells (Drobna et al. 2010; Liu et al. 2006), as well as mouse studies on organ-specific distribution, retention, and excretion of specific metabolites (Kenyon et al. 2008), have appeared. MMA species can accumulate in cells (perhaps owing to their reactivity), whereas DMA is readily exported. More genetically amenable models are now available for study. Arsenite-fed *AS3MT*-knockout mice produced low levels of methylated metabolites but accumulated high levels of iAs (up to 20-fold higher than wild-type mice) in various tissues (Drobna et al. 2009; Hughes et al. 2010), supporting methylation as a key pathway for arsenic elimination. Such iAs accumulation led to early death (Yokohira et al. 2010).

Organisms such as *Drosophila* and yeast are simpler eukaryotes that have genetic advantages and few confounders. These organisms provide

experimentally accessible models capable of rapidly generating fresh insight and testable hypotheses. Thus, *Drosophila* lacks a homolog of *AS3MT*, but introducing the human *AS3MT* gene allows both MMA and DMA species to be produced. Arsenite-fed transgenic flies show important dose-dependent differential effects of these species *in vivo* compared with the wild-type, with significantly impaired chromosomal stability at 9 ppm but enhanced viability at an acute exposure of > 60 ppm owing to reduced arsenic accumulation (Muñiz Ortiz et al. 2011). The data integrate the idea that methylated arsenicals are more damaging to macromolecules yet are more readily eliminated and that iAs dose makes all the difference to phenotypic consequence. Importantly, the quantitative consequences of other human *AS3MT* alleles (e.g., M287T) can be tested readily in this system. The availability of a transcriptome-wide RNA interference-based gene knockdown system in *Drosophila* should provide novel screens that identify pathways intersected by such metabolites. Complementary approaches already initiated in yeast using a gene deletion library have identified novel pathways pertaining to arsenite methylation and histone H4 methylation that are relevant in human cells (Jo et al. 2009; Ren et al. 2011a).

Disease Outcome Dependence on Interaction with Genetics and Other Environmental Factors

Humans exposed to arsenic do not all succumb to a single disease. Some develop cancer, whereas others develop cardiovascular disease or neuropathies. The reason for the different responses to similar exposures is unclear. A hint is apparent in the differential response of different strains of mice to similar *in utero* arsenic exposures. C3H, CD, or Tg.AC mice develop earlier and more severe cancer (Tokar et al. 2011; Waalkes et al. 2003, 2008), whereas ApoE$^{-/-}$ mice develop earlier and more severe atherosclerosis (Srivastava et al. 2007, 2009). These responses are clearly linked to the disease predisposition of the mice, and this disposition appears to be aggravated by the arsenic exposure. Thus, arsenic interaction with the genetic background of the organism determines the disease outcome in these models. In humans, disease outcome also is likely dependent on interaction with other exposures in addition to individual genetic predisposition. Immunosuppression by arsenic exposure may increase susceptibility to infectious agents (Kozul et al. 2009). Thus, increased sensitivity to viral infections could increase oncogenesis if the individual is exposed to oncogenic viruses such as HPV. Chronic arsenic exposure causes hyperreactivity to lipopolysaccharide (Arteel et al. 2008), suggesting that aggravated inflammatory responses to bacterial infections or even to nonpathogenic exposures could be aggravated. Hence, arsenic exposure may prime the system for exaggerated response to a second hit that could be a biological or physical agent, diet, or altered metabolism encoded by individual genetics. Thus, more studies both of human genetics and disease outcome and of structure/function relationships of polymorphic genes involved in arsenic metabolism are needed.

Conclusions

Chronic arsenic exposure, mostly via contaminated drinking water, causes a multitude of diseases. It is unclear what governs the specific pathology induced in any given individual. However, genetic susceptibility to a particular disease and interaction with other environmental factors play major roles in determining disease outcome of arsenic exposure. Anchoring of experimental models for arsenic toxicology to specific human exposures is essential to gaining a mechanistic understanding of how arsenic exposure leads to specific human pathologies. Global gene expression profiling, epigenetic mapping, and markers of tissue remodeling offer promise as phenotypic anchors. Full development of anchors requires extensive research to profile gene expression, to map epigenetic marks, and to identify biomarkers in target, or surrogate, tissues in arsenic-exposed populations. Human research that includes dosimetry would have greatest impact.

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